

# **Development of proxy indicators for methane output by sheep using rapid-throughput field and laboratory techniques**

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**A thesis submitted in partial fulfilment of the requirements for  
the degree of:**

**Doctor of Philosophy (Ph.D.) in Biological Sciences**

**at the**

**Institute of Biological, Environmental and Rural Sciences  
(IBERS), Aberystwyth University**

**2015**

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## **Acknowledgements**

I am extremely grateful to the English Beef and Lamb Executive (EBLEX) and Hybu Cig Cymru (HCC) for funding this Ph.D. project and providing numerous opportunities for me to present and discuss my work with others working in similar areas of research.

I would also like to thank my supervisors, Dr. Jon Moorby and Professor Jamie Newbold for their advice during my Ph.D. I would particularly like to thank my main supervisor, Dr. Jon Moorby for his invaluable help, support and encouragement throughout my Ph.D. project and write-up period.

I am also very grateful to all of the IBERS staff who helped me and taught me experimental methods and how to identify plant samples, particularly Hannah Fleming, Vince Theobald, Dave Leemans and Jim Vale.

## Summary

Methane production by ruminants is a significant contributor to agricultural greenhouse gas emissions (Webb *et al.*, 2013). However, current values used to estimate methane output by sheep are default values and do not take into account animal and dietary factors that may affect methane output (Bernstein *et al.*, 2007). Strategies to reduce ruminant methane output are the focus of a large body of research (Iqbal *et al.*, 2008) and, in order to implement these strategies fully, a greater understanding of factors that influence ruminant methane emissions is necessary.

The "gold standard" method for measuring methane output by sheep is the use of respiratory chambers (Blaxter and Clapperton, 1965). However, this method is expensive, time-consuming and labour intensive, making it unsuitable for use in an on-farm situation. The work presented in this thesis explores the potential of three proxies to estimate methane output by sheep, which could be used or adapted to be used as a practical means of estimating methane emissions from sheep on a large scale.

The proxies investigated here are a Laser Methane Detector (LMD), used to take measurements of methane concentration from air expired by sheep, *in vitro* gas production analysis of feeds offered to sheep, and Fourier-transform infrared spectroscopy (FTIR) analysis of feeds offered to sheep. Predictions of methane output obtained from each of the proxies are validated using respiratory chamber measurements taken from sheep offered a variety of feeds during different experiments.

With further development and validation, all three proxies presented in this thesis demonstrate potential to be used to successfully estimate or predict methane output by sheep as measured in respiratory chambers.

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# Abstract

## Introduction

Methane is a potent greenhouse gas with a global warming potential of 34 times that of carbon dioxide over a 100 year period (Stocker *et al.*, 2013). Enteric methane production by ruminants is a significant contributor to agricultural emissions, which accounted for 8.44% of total UK greenhouse gas emissions in 2011 (Webb *et al.*, 2013). Ruminant methane production may also be associated with economic losses for farmers: 2–15% of ingested energy is used to produce methane in the rumen (Giger-Reverdin *et al.*, 2000). Theoretically, this energy could be used to increase animal production. Through livestock management, there may be opportunities to reduce enteric methane emissions (Gerber *et al.*, 2013). However, a greater understanding of the causes of variation in methane emitted by individual animals is necessary to facilitate the introduction of new strategies for reducing ruminant methane production. Current estimates of methane output by sheep are based on default values, with little differentiation based on the characteristics of individual sheep and production systems. The aim of this Ph.D. project was to identify, develop and validate methods that could be used to estimate methane emissions by sheep at a large on-farm scale.

## Methods

Following a review of the current relevant literature, three methods were identified as potential proxy indicators for methane output by sheep: the use of a Laser Methane detector (LMD; Tokyo Gas Engineering Ltd., 2006), *in vitro* gas production using animal feed samples, and Fourier-transform infrared (FTIR) spectroscopy using animal feed

samples. A series of experiments was conducted, concurrent with work being done as part of the Defra-funded project AC0115, in which methane output of sheep of four different breeds was measured using respiratory chambers. This form of measurement is considered the "gold standard" method for measuring methane output by sheep. The daily methane output data obtained in this series of experiments was, therefore, used to validate all proxy methods investigated in this project. During the respiratory chamber experiments, LMD measurements were taken from individual sheep prior to entering chambers. The methane concentration data collected using the LMD was used to calculate approximate daily methane output by each sheep, which were then compared with the daily methane output data for individual sheep as calculated using the respiratory chamber data. Feed intake was measured in all chamber experiments and feed samples were taken and freeze-dried to be used for *in vitro* gas production and FTIR spectroscopy. For *in vitro* gas production, feed samples were incubated with rumen fluid obtained from ruminally fistulated cows. Total gas production and methane concentration in the gas produced were measured, allowing for the calculation of methane produced per gram of each feed sample. This was then compared with methane output by sheep per unit of feed intake. Using the same feed samples, FTIR spectroscopy was conducted. Partial least squares (PLS) regression was used to determine whether a model derived from the FTIR spectra of feed samples could be used to predict methane outputs by sheep given different feeds as measured using the respiratory chambers.

## **Results**

The daily methane outputs calculated using the LMD were in the range that would be expected from sheep. Furthermore, these estimates significantly correlated with those



calculated using the chamber data in all experiments, although the LMD results had a tendency to underestimate methane output as compared with chamber data. Significant correlations were also found between estimates of methane output per unit of feed obtained from *in vitro* gas production data and methane output per unit of feed intake measured using respiratory chambers. The methane production profiles of the different feeds produced using *in vitro* gas production were easily and consistently distinguishable, with little overlap of standard errors. Seventeen PLS components were identified using PLS regression of FTIR data, which accounted for 100% of the variation in methane outputs obtained from respiratory chamber measurements. Cross validation was conducted and four PLS components was considered to be the optimum number to use in the PLS model to avoid over-fitting. Using this model, FTIR data from feed samples could successfully predict methane outputs of sheep fed on different diets as measured using respiratory chambers.

## **Discussion**

All three proxies investigated as part of this Ph.D. project (the LMD, *in vitro* gas production and FTIR spectroscopy) demonstrated potential to estimate or predict methane output by sheep as measured using the respiratory chambers. A novel and very successful approach to the method for use of the LMD and calculation of daily methane emissions from LMD data is presented in this thesis. However, the methods used were relatively labour intensive and time-consuming. Further work should, therefore, focus on simplifying these methods as much as possible. To my knowledge, the results presented for *in vitro* gas production and FTIR spectroscopy are also novel, although these are established methods. Both of these methods are rapid-throughput techniques and, therefore, have real potential to be used on a large scale. Further

work using larger data sets may provide a more comprehensive idea of the aspects of feeds that affect their methane potentials.

## Chapter 1

### Introduction and Review of the Literature

# **1 Introduction and review of literature**

## **1.1 Methane production in ruminants**

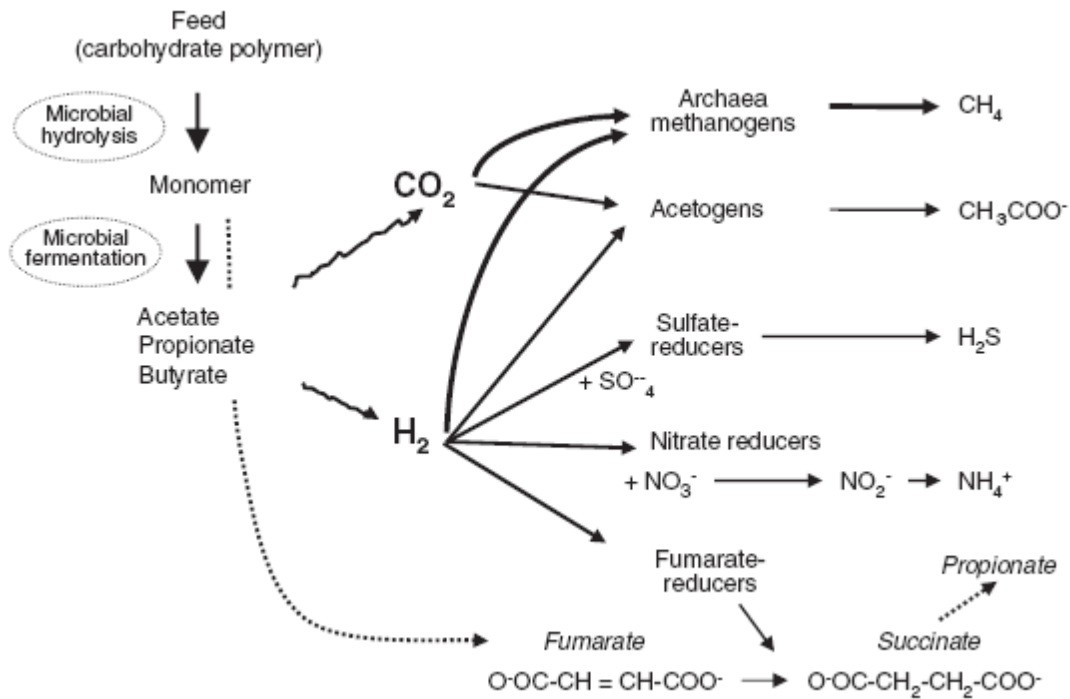
### **1.1.1 Introduction**

The rumen acts as a fermentation chamber in ruminants, allowing the digestion, by microbial enzymes of those components of the diet that cannot be digested by mammalian enzymes. The microbes present in the rumen are largely anaerobic bacteria, protozoa, archaea and anaerobic fungi. In general, ruminants spend approximately one third of their time grazing and another third ruminating: this is the regurgitation and re-chewing of digesta to allow smaller particles to pass into the omasum, the next part of the digestive tract. Carbohydrates, including fibrous carbohydrates, are fermented in the rumen to allow the growth of microbial cells and other products, including volatile fatty acids (VFAs), carbon dioxide and methane ( $\text{CH}_4$ ). Ruminant physiology has evolved to make use of VFAs and other fermentation products to provide energy. Lactate, ethanol, succinate and hydrogen are intermediate products of ruminal fermentation. Lactate and ethanol are converted into acetate and succinate is converted into propionate. Hydrogen, on the other hand, is used as an energy substrate by methanogens, producing methane as a by-product. As methane cannot be used by ruminants, methane production effectively decreases the energy available to the animal, as hydrogen can also be used to produce propionate, which would be a useful energy substrate (Hungate, 1975).

### **1.1.2 Mechanism of methane production in the rumen**

Methanogenesis occurs in the rumen due to the presence of methanogenic archaea, which inhabit anaerobic environments, such as the rumen. Five species of

methanogenic archaea, belonging to the genera *Methanobrevibacter* and *Methanosarcina*, have been isolated from rumen digesta (Moss *et al.*, 2000). Hydrogen and carbon dioxide are the substrates used by methanogenic archaea to produce methane. Hydrogen and carbon dioxide are both end products of microbial fermentation in the rumen; that is, the hydrolysis of carbohydrate monomers to form VFAs (acetate, propionate and butyrate). Once released by the formation of VFAs, hydrogen and carbon dioxide are either converted into methane by the methanogenic archaea or acetate by acetogens. Some hydrogen is also used in the formation of hydrogen sulphide by sulphate reducers, ammonium ions by nitrate reducers, or in the conversion of fumarate, another product of microbial fermentation, to succinate and, eventually, propionate (Morgavi *et al.*, 2010). Feed additives that divert hydrogen and/or carbon dioxide away from the methanogenesis pathway and into any of these other pathways have potential use in the reduction of methane emissions from ruminants (Morgavi *et al.*, 2010). The various pathways for the use of hydrogen and carbon dioxide are shown in Figure 1. Methanogens have a lower threshold for hydrogen than acetogens and, therefore tend to outcompete them; methane is produced preferentially to acetate, which could be used as an energy substrate (Liu and Whitman, 2008). In sheep, 95% of methane produced in the rumen is excreted via eructation. Approximately 89% of methane produced in the lower gut, which represents about 13% of total methane production, is absorbed from the gut and exhaled from the lungs. Therefore, about 98% of methane produced by sheep is excreted through the mouth and nostrils (Murray *et al.*, 1976).



**Figure 1: Diagrammatic representation of pathways of hydrogen and carbon dioxide utilisation in the rumen (Morgavi et al., 2010).**

## **1.2 Methane as a greenhouse gas (GHG)**

### **1.2.1 Climate change: causes and consequences**

The Intergovernmental Panel on Climate Change (IPCC) Synthesis Report (2007) defines climate change as 'any change in climate over time, whether due to natural variability or as a result of human activity' (Bernstein *et al.*, 2007). There is undeniable evidence of global warming in the last fifty years, including rising average air and sea temperatures, melting of snow and ice, and rising global sea levels (Bernstein *et al.*, 2007). There is strong evidence that global warming is having a significant impact on natural systems based on snow and ice, as well as terrestrial ecosystems; for example, increases in numbers and size of glacial lakes, decreased ground stability in mountainous areas, with increased risk of avalanches, early spring vegetation growth and changes in bird migratory patterns (Bernstein *et al.*, 2007). Marine and freshwater systems have also undergone rises in water temperature, as well as changes in ice cover, salinity, oxygen levels and circulation, which has affected numbers of fish, algae and plankton in different ecosystems, as well as affecting fish migratory patterns (Bernstein *et al.*, 2007). There is evidence that human systems, including agriculture and forestry management, are affected by global warming, including earlier planting of crops and increased incidence of forest fires (Bernstein *et al.*, 2007). Human health may also be affected by increases in global temperatures, with disease vectors, such as mosquitoes carrying malaria, becoming more widespread. Incidence and frequency of extreme weather events, such as heat waves and heavy precipitation, have also increased in the last fifty years; this is likely to be a result of global warming (Bernstein *et al.*, 2007). Therefore, climate change is a significant threat to many natural and human systems.

Although there may be natural variability in climate change, there are also anthropogenic causes of global warming, which it is important to monitor and mitigate. Greenhouse gases (GHGs), including carbon dioxide, methane, nitrous oxide, water vapour and ozone, are present in the Earth's atmosphere; these are gases that absorb and emit solar radiation in the thermal infrared range, trapping it within the atmosphere and thereby increasing the temperature of the Earth. This is known as the Greenhouse Effect. Anthropogenic activities, such as burning of fossil fuels, increase the concentrations of these gases in the atmosphere, increasing the Earth's temperature and leading to climate change.

### **1.2.2 Economic losses associated with ruminant methane production**

Although the main focus of current research is reducing the environmental impact of ruminant livestock on the environment, a reduction of methane output by animals would also have economic benefits (Iqbal *et al.*, 2008). This is due to the energy being used in the production of methane in the rumen: Giger-Reverdin *et al.* (2000) estimate that 2-15% of ingested gross energy is used for methane production, which is not used by the animal and, therefore, represents an energy loss. Theoretically, the energy used to produce methane in the rumen could be used to increase animal production without increasing energy intake. For example, by feeding cattle grain rather than forage, the percentage of energy used that is converted to methane has been shown to be reduced from 6.5% to 3% (Beauchemin & McGinn, 2005). However, this would require changes to the animal's metabolism, favouring propionate production over acetate and butyrate production (see Figure 1). Dietary additives and alterations in diet have the potential to alter the metabolism in this way, although to date, few *in*



*vivo* studies have examined the long-term effects of mitigation agents (Hristov *et al.*, 2012).

### **1.2.3 Targets to reduce GHG emissions**

The United Nations Framework Convention on Climate Change (UNFCCC) implemented the Kyoto protocol in 1997; this is an international agreement in which participating countries commit to GHG emission reduction targets. The Climate Change Act (2008) sets a UK target to reduce GHG emissions by 80% by 2050 from a baseline of emissions from 1990. The act also states that there must be an annual statement (inventory) of GHG emissions in the UK.

Methane is one of the GHGs targeted by the Climate Change Act (2008). Methane follows carbon dioxide as the second most significant GHG in the UK and globally, representing 14.3% of global anthropogenic GHG emissions (Bernstein *et al.*, 2007). The global warming potential (GWP) of methane is approximately 34 times that of carbon dioxide over a 100 year period (Stocker *et al.*, 2013). Between 1990 and 2011, total UK methane emissions (from all sectors) decreased by 57.3%, and UK methane emissions from the agricultural sector decreased by 20.6% (Webb *et al.*, 2011). However, in 2011, there were still methane emissions of 42Mt of carbon dioxide equivalent (Webb *et al.*, 2013).

### **1.2.4 Agricultural GHG emissions**

Agriculture is a major source of GHG emissions in the UK; it was responsible for 8.44% of total UK GHG emissions in 2011 (Webb *et al.*, 2013). Methane emissions from agriculture decreased by 20.6% between 1990 and 2011 (Webb *et al.*, 2013). This is largely due to a reduction in livestock numbers, reducing the methane produced by

enteric fermentation, which accounts for approximately 39% of GHG emissions from the agricultural sector (Gerber *et al.*, 2013a). However, there is a strong relationship between productivity and emission intensity (emissions per unit of production; e.g. methane per kg live weight gain) in ruminant production systems; emission intensity decreases as yield increases. Therefore, through livestock and manure management, there are possibilities to reduce methane emissions from the agricultural sector still further (Gerber *et al.*, 2013).

### **1.2.5 Methane emissions by sheep**

Small ruminant (sheep and goat) supply chains for meat and milk are responsible for about 6.5% of global GHG emissions from the agricultural sector (Gerber *et al.*, 2013a). Approximately 55% of these emissions are the result of enteric fermentation (Gerber *et al.*, 2013a). Default Tier 1 IPCC (2006) emission factors (EF) estimate methane emissions by sheep to be 8kg/head/year in developed countries and 5kg/head/year in developing countries. Tier 1 emissions estimates are the simplest form of emission estimate, in which a default value is used for an emission factor. Tier 2 estimates are those that use country specific emission factors and other data, and Tier 3 estimates use complex modelling approaches. Factors including body weight, dry matter intake, breed, diet and production system may affect methane produced by individual sheep. Tier 1 estimates do not take into account any of these factors. There is, therefore, potential to improve the accuracy of emissions estimates for sheep. Proxy indicators may be instrumental in developing Tier 2 and 3 estimates for methane output by sheep.

## **1.3 The problem of measuring methane from animals**

### **1.3.1 Variation in ruminant methane production**

The factors that affect methane production in the rumen include the amount and type of feed ingested, changes and variation in ruminal microflora, feed processing and feed additives, such as lipids or ionophores (Johnson and Johnson, 1995). Methane losses from the gross energy (GE) intake are highly variable, ranging between 2 and 12% (Johnson and Johnson, 1995). This variability is due to the proportions of different VFAs produced during ruminal fermentation (Johnson and Johnson, 1995). These VFAs provide roughly 80% of a ruminant's dietary requirement for energy (Hart *et al.*, 2008), but certain ratios of acetic: propionic acid would result in energy losses of up to 33% of the GE intake due to methane production, though these ratios do not occur in practice: theoretically, at a ratio of 0.5, 0% of the GE is lost as methane, whereas methane losses of 33% occur if all carbohydrate is fermented to acetic acid (Johnson and Johnson, 1995). In practice, the ratio of acetic: propionic acid can vary between 0.9 and 4, hence the wide variation in methane losses. As a high proportion of propionic acid results in a reduction in ruminal methane production, it has been suggested that feed additives, such as fish oil, which reportedly increases ruminal propionic acid concentration, therefore reduce methane production in the rumen (Fievez *et al.*, 2003).

It has been shown that there are statistically significant differences in methane production between animals given the same diet; these differences could not be entirely explained by difference in breed; animals of the same breed given the same diet exhibited individual significant differences in methane production (Blaxter and

Clapperton, 1965). There appears to be some natural variation in methane production between animals: Goopy *et al.* (2006) found that although some animals exhibited an increased methane yield with increased dietary intake, differences in methane production between animals classified as “low” methane yielding and those classified as “high” methane yielding remained significant, regardless of dietary intake. The percentage of propionate from the total VFA yield in the rumen was shown to be higher in animals classed as “low” methane yielding than in those classed as “high” methane yielding. This conforms to the idea that the pathway for production of propionate is competitive with that for methane production (Moss *et al.*, 2000). The inter-animal variation in methane production demonstrates that there are genetic factors involved in the rate of methanogenesis, possibly caused by permanent differences between microbial populations of different animals (Hegarty *et al.*, 2007). Many methanogens in the rumen are associated with the rumen protozoa: Newbold *et al.* (1995) reported a significant reduction in methane production from defaunated (i.e. without rumen protozoa) animals compared to control animals. Belanche Gracia *et al.* (2011) also found that inoculation of defaunated sheep with holotrich protozoa increased methane emissions by approximately 65%, without substantially modifying rumen fermentation patterns but increasing ruminal acetate/propionate and propionate/butyrate ratios. Protozoal activity may be modulated by an immune response (Gnanasampanthan, 1993); perhaps, therefore, individual differences in microbial populations are due to differing levels of immune response to the rumen protozoa.

### **1.3.2 Persistence of methane emissions from individual animals**

The persistence of methane output by animals over time is an important consideration when attempting to quantify livestock methane emissions. There is conflicting evidence concerning the persistency of methane output by individual animals. Goopy & Hegarty (2004) and Münger & Kreuzer (2008) found that methane emissions from the same animals may not persist over time. However, Goopy *et al.* (2006) found that although methane yield of individual animals varied over time, those which were originally classified as high or low yielding remained so over time; inter-animal differences in propionate concentration and protozoal density also persisted over time. Though there may be some genetic effects on methane yield by animals, all three studies agreed that dry matter intake and diet variation were important factors, which should be taken into account.

### **1.3.3 Diet-dependent variation in methane production**

Despite the apparent natural variation in methane production between animals, Blaxter and Clapperton (1965) found that there was considerable variation in methane production associated with feed in the same animals: the lowest average methane production recorded was from animals offered a diet of pelleted meadow fescue grass and the highest was from animals offered a diet of sugar beet pulp. In general, it was concluded by Blaxter and Clapperton (1965) that methane production increased with increased feed digestibility at a maintenance level of feeding, though methane production decreased when animals were fed highly digestible feed at three times maintenance levels. Smaller amounts of more highly digestible feeds are required to meet animal maintenance needs than relatively less digestible feeds, and there is a

positive correlation between feed intake and methane production; feeding less digestible feeds could increase feed intake and thereby methane production. However, there is a well-established relationship between increased digestibility and increased voluntary intake (Baumont, 2000). Therefore, unless feed is restricted, highly digestible feeds may increase intake and thereby increase methane emissions. Diet has a considerable influence on methane production levels and has the potential to be manipulated in order to reduce methane emissions from ruminants. When investigating potential proxy indicators for methane production, therefore, considering the methane potentials of feeds is important.

#### **1.3.4 The need to improve the accuracy of emissions factors for sheep**

The variation in methane production between animals of the same breed and fed the same diet, as demonstrated by Blaxter and Clapperton (1965) and Goopy *et al.* (2006), creates a problem in attempting to estimate the methane production of a particular animal or group of animals. With increasing pressure on governments to reduce greenhouse gas emissions from agriculture, following the Kyoto protocol (1997), it is necessary to be able to give an accurate estimate for the methane production per animal. Although there is inter-animal variation in methane production, there is potential to develop more accurate ways to estimate methane emissions based, for example, on proxies such as residual feed intake, taking into account the type of feed given, or even body mass. It has been suggested that a way of mitigating methane emissions would be to selectively breed animals, selecting for reduced methane production (Kebreab *et al.*, 2006). However, according to Bernstein *et al.* (2007), it is not currently possible to establish such a breeding programme due to the scale upon

which methane emissions would need to be accurately measured. The development of an accurate proxy for the estimation of methane emissions could therefore be of considerable use with regard to selecting for low ruminal methane production. Any attempts to introduce national or global incentives or programmes for the use of methane mitigation treatments would only be viable if more accurate ways of predicting methane emissions are available so that treatments can be taken into account in the estimation of methane production by a particular animal or group of animals.

As mentioned in Section 1.2.4, the emissions factors used by Bernstein *et al.* (2007) for sheep are based on Tier 1 methodology, not taking into account country-specific data regarding nutrient requirements, feed intake and methane conversion rate for specific feed types (Gibbs *et al.*, 2000). The collection of the necessary data is more challenging for the Tier 2 system, though Bernstein *et al.* (2007) recommend the development of this system for countries with large numbers of ruminant animals. The other potential cause for inaccuracy, even using the Tier 2 system, is the reliability of data collection methods in each country, although this is considered by Gibbs *et al.* (2000) to be a lesser cause of inaccuracy. Tier 3 methodologies are effectively models that calculate methane emissions based on estimations such as energy requirements and feed intake of various ruminant species and breeds at different physiological states. Comparing the Tier 3 system with the Tier 1 system, Woods and Yan (2010) found that, overall, greenhouse gas emissions from ruminants were calculated to be 5% lower using the Tier 3 system than the Tier 1 system, though, for some classifications of animals, the Tier 3 system gave higher values for emissions. Woods and Yan (2010) argue that there

is a need for emission factor data that is more representative in terms of the age and diet of the animal.

Kebreab *et al.* (2008) state that mechanistic models of methane production in ruminants give a more accurate estimation of emissions than any of the IPCC empirical models: IPCC values were found to overestimate the methane production of dairy cows by 12.5% and to underestimate methane production of feedlot cattle by 9.8%. Mechanistic models tend to be more accurate as they are diet-specific. The difference between empirical and mechanistic models is that empirical models attempt to relate nutrient intake directly to methane output using established data, whereas mechanistic models aim to use ruminal fermentation biochemistry to simulate methane emissions (Kebreab *et al.*, 2008). Kebreab *et al.* (2008) argue that any attempt to create incentives for the mitigation of methane emissions would require the more accurate estimates of methane production that could be obtained by diet-specific mechanistic models.

As demonstrated in this section, there is considerable variability in methane production by different animals and in different production systems. This section also highlights that there is potential to improve emissions estimates for sheep, which are currently calculated in the UK using the simplest Tier 1 methodology. Proxy indicators for methane output by sheep could be instrumental in achieving more accurate emissions factors for sheep.



## **1.4 Methods of estimating methane emissions from sheep and potential proxies for estimating these methane emissions**

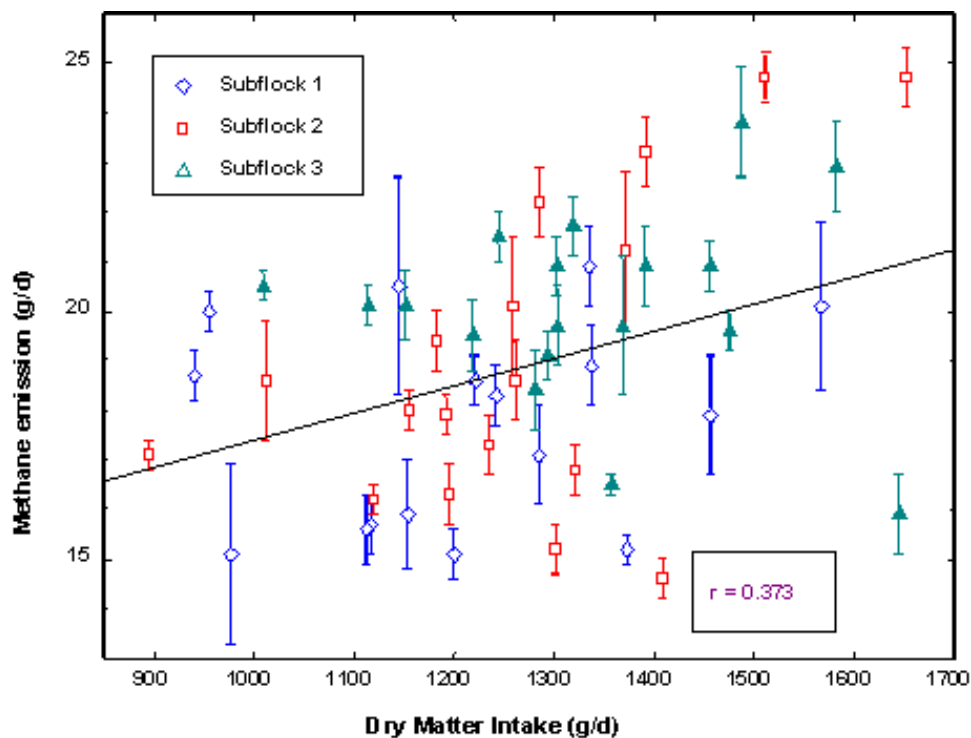
### **1.4.1 *In vivo* methods**

*In vivo* methods for estimating methane output by ruminants include the relationship between dry matter (DM) intake and methane production, open-circuit respiration chambers, the sulphur hexafluoride (SF<sub>6</sub>) tracer technique, micrometeorological techniques, the use of a hand-held laser methane detector (LMD), and sampling during feeding. These methods, along with their advantages and limitations, are described in this section.

#### **1.4.1.1 Body weight, dry matter intake and passage rate through rumen**

Moe and Tyrrell (1979) found that reasonable predictions of methane emissions could be made using total carbohydrate intake but, for more accurate estimations, the type of carbohydrate should be taken into account. It was found that the most useful predictions of methane production could be obtained by measurement of the amounts of soluble residues, hemicellulose and cellulose digested. These carbohydrates are fermented in the rumen as mammalian enzymes are incapable of hydrolysing them; they are therefore digested by microbial enzymes, leading to hydrogen release and availability for methanogenesis. Lassey *et al.* (1997) found a correlation, shown in Figure 2, between dry matter (DM) intake and methane emissions from sheep. However, the correlation is weak ( $R=0.373$ ), indicating that DM intake is not accurate on its own as a proxy unless factors such as carbohydrate type and digestibility are taken into account. Molano & Clark (2008) demonstrated a stronger correlation between DM intake and daily methane emissions ( $R^2=0.83$ ), suggesting that DM intake could accurately predict methane emissions. However, even

after adjusting methane emissions for DM intake, there is evidence to suggest that there is genetic variation between animals in terms of methane output (Pinares-Patiño *et al.*, 2013), indicating that DM intake cannot be fully relied upon as a measure of methane output.



**Figure 2: Relationship between DM intake and body weight (Lassey *et al.*, 1997)**

A difficulty arises in that, particularly for grazing animals, it is not feasible to accurately measure carbohydrate intake. Body weight would be an extremely quick and simple way to estimate methane output if it could provide an accurate estimate. However, the results of experiments using sheep of varying weights show that, when fed *ad libitum*, body weight does not accurately predict either dry matter intake or methane output as measured in calorimetric chambers (Fraser *et al.*, 2013). In intensive systems, in which it is possible to measure or estimate dry matter intake, it may be possible to use this measurement to predict methane emissions from animals.

However, in grazing situations, which are common for sheep in the UK, it is not possible to measure dry matter intake, and body weight may not be a sufficiently accurate indicator of dry matter intake to be useful in predicting methane emissions. Safari *et al.* (2005) investigated the potential of weight of wool produced by sheep to estimate methane production. There was, however, no significant correlation between wool weight and methane emissions and, when the results were adjusted for body weight, the correlation was close to zero.

There has also been some investigation into the relationship between passage rate of feed through the rumen and methane production. A faster passage rate allows less time for microbial fermentation in the rumen, reducing the availability of hydrogen and, therefore, the potential for methane to be produced (Johnson and Ward, 1996). Pinares-Patiño *et al.* (2003) found that methane production by sheep, as measured using the sulphur hexafluoride ( $\text{SF}_6$ ) tracer technique (Johnson *et al.*, 1994) decreased as passage rate, measured using Cr-EDTA and lignin as markers, increased. However, measuring passage rate through the rumen is complicated and only feasible in an experimental situation.

#### **1.4.1.2 Open-circuit respiration chambers**

An open-circuit respiration chamber method was first used to measure methane from sheep by Blaxter and Clapperton (1965) and chamber methods are still widely accepted to be the most accurate means to measure methane emissions by ruminants. Chamber techniques have been used extensively to validate other, less well established methods for methane measurement, such as the sulphur hexafluoride ( $\text{SF}_6$ ) tracer technique (Johnson *et al.*, 1994) and micrometeorological techniques

(Grainger *et al.*, 2007; Pinares-Patiño *et al.*, 2008; Tomkins *et al.*, 2011). Chamber design can vary between institutions; the basic principle is that ambient air is circulated around the animal and expired air is collected. Methane emissions are calculated by measuring the total air flow through the chamber and the difference in methane concentration between the ambient and expired air (Johnson & Johnson, 1995). Although the nature of chambers requires some restriction to an animal's normal behaviour and movement, animals are free to move within chambers and their behaviour should not be particularly abnormal (Johnson & Johnson, 1995). Chambers must first be calibrated using gases released at a known rate and concentration. Once calibrated, methane recovery tests could be performed. This involves releasing a standard methane gas into chambers at a rate that was similar to methane production by sheep. Klein and Wright (2006) showed that between 94.4 and 107.1% of methane was recovered using the chamber technique. Chamber methods can, therefore, provide accurate measurements of methane from individual animals.

There are, however, some limitations to the chamber method. To obtain daily methane measurements, animals are kept in chambers for two or three days, making this a very time consuming method. The expense and practicalities of installing and using chambers makes them impractical for use on a large on-farm scale. Chamber measurements of methane emissions might not reflect methane emissions from the same animals when grazing; being in the methane chambers may affect factors such as feed intake, which would have considerable impact on methane production. However, in the absence of reliable alternatives, methane chamber methods remain the 'gold standard' for calculation of methane emissions by ruminants. They may be useful in

the development of more accurate methane emissions factors and for the validation of proxy indicators for methane output.

#### **1.4.1.3 Portable static chambers**

A shortcoming of open-circuit respiration chambers is that they cannot be used in grazing situations and, while they can provide accurate repeatable measurements of methane emissions by animals, they may affect behaviour, particularly eating habits. This brings into question whether or not the daily methane emissions obtained using chambers are truly representative of methane production by grazing animals.

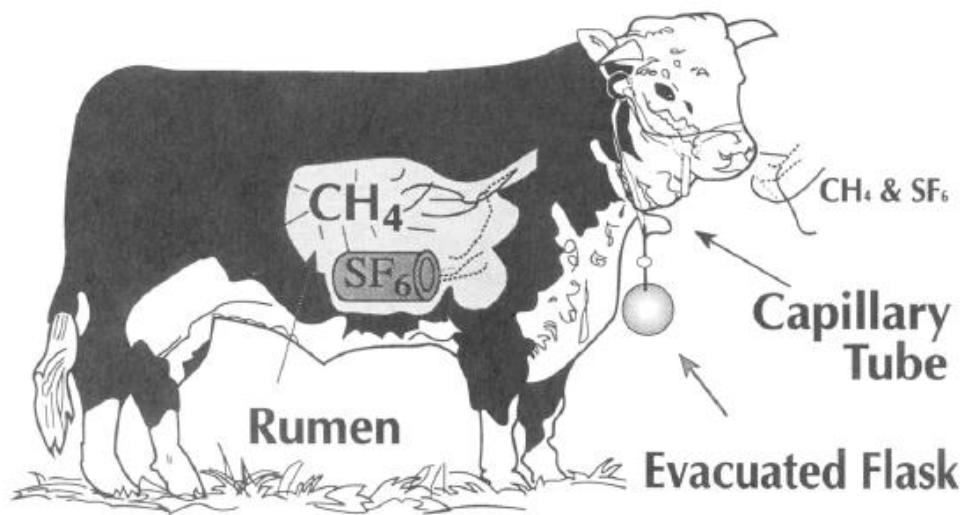
Portable static chambers, which can be used in field situations, may provide an alternative form of methane measurement that does not interfere with normal grazing activity. Goopy *et al.* (2011) experimented using short (one hour) measurements in portable static methane chambers for sheep. These chambers were designed for one or two hour methane emission measurements and showed a low gas leakage, with 98-99% of an injected tracer gas remaining after two hours. Methane emissions as measured over one hour periods in the portable static chambers significantly correlated ( $R=0.71$ ) with average methane emissions measurements taken from the same sheep over 22 hour periods in open-circuit methane chambers. Portable static chambers could, therefore, provide a means of estimating methane emissions from animals in grazing situations. Shorter measurements of one or two hours could also be effective as representations of methane emissions over the course of a day (Goopy *et al.*, 2011).

#### 1.4.1.4 Sulphur hexafluoride (SF<sub>6</sub>) tracer technique

The sulphur hexafluoride (SF<sub>6</sub>) tracer technique was developed by Johnson *et al.* (1994) to measure methane emissions from individual grazing animals, and is shown in Figure 3. The technique involves the placement of a permeation tube containing SF<sub>6</sub> in the rumen, which is released at a known and constant rate, and measuring concentrations of SF<sub>6</sub> and CH<sub>4</sub> near the mouth and nostrils of the animal. The assumption upon which the method is based is that dilution rates of SF<sub>6</sub> and CH<sub>4</sub> are identical when they reach the mouth, and by knowing the rate of SF<sub>6</sub> release, the rate of methane emission can be calculated. According to Johnson *et al.* (1994), the implications of this method of methane measurement are that it provides a simple way to obtain a large database of livestock methane emissions that will, in turn, give greater knowledge of the contribution of ruminants to methane emissions. The tracer technique also allows the animal to be grazing under more normal conditions rather than being confined to a chamber, which may have an impact on methane emissions results.

The technique is now widely used in studies that aim to measure methane emissions by grazing animals (Lassey, 2007). Lassey *et al.* (2007) found that there was good consistency in inferred daily methane emissions from sheep, as measured using the SF<sub>6</sub> technique, when repeated 24 hour samples were collected over a five day period. However, Vlaming *et al.* (2008) found high within-animal variability in methane measurements, as measured using the SF<sub>6</sub> technique, causing difficulties in obtaining consistent rankings between animals in terms of methane yield. Vlaming *et al.* (2008) also suggest that the technique may exaggerate apparent inter-animal differences in methane emissions. More recently, however, Lassey *et al.* (2011) found that the tracer

technique was a reliable and unbiased method for the measurement of methane emission rate in ruminants. This study also highlights that the technique is currently the only one viable for measuring emission rates from individually grazing animals, though further work may be required to improve and validate the technique.



**Figure 3: Diagram demonstrating the  $\text{SF}_6$  technique (Johnson *et al.*, 1994).**

#### **1.4.1.5 Micrometeorological techniques**

Micrometeorological techniques, including mass balance techniques, are methods for providing methane emissions estimates on a large scale. Mass balance techniques can be used to estimate methane emissions from barns by measuring the methane concentration at the barn inlet and outlet and calculating the emission rate using the outlet ventilation rate (Harper *et al.*, 2011). This is a simple method that uses a similar principle to methane chambers, but is not considered accurate or representative, due to factors such as air samples being dirty or moist and spatial gradients in interior concentrations (Harper *et al.*, 2011). Also, for the purposes of estimating methane emissions by sheep, which are more commonly in a grazing situation rather than in a barn, this method is not very suitable. Lockyer and Jarvis (1995) used the principle of the mass balance technique in using large poly tunnels to estimate methane emissions from grazing animals. The estimates were effected, in this case, by whether animals settled and grazed evenly in the tunnel or not.

Modified mass difference techniques do not require a spatially uniform area methane source and do not rely on the assumptions about atmospheric transport that are present in mass balance methodologies (Harper *et al.*, 2011). This method involves measuring the gas flux across each face of a rectangular control volume that surrounds the methane source. A drawback to the method is the requirement for methane concentration measurements at many different points. These measurements can be made by surrounding paddocks with porous tubing (Harper *et al.*, 1999) or using open-path laser spectrometry (Desjardins *et al.*, 2004). Tomkins *et al.* (2011) compared a micrometeorological method with open-circuit respiration chambers to determine methane emissions from beef cattle. The micrometeorological method used involved



atmospheric turbulence data and methane concentration measured using an open-path laser. This method overestimated methane emissions as measured in open-circuit respiration chambers. Micrometeorological techniques may be useful for estimating emissions from groups of animals, particularly when animals are housed in barns and paddocks. These techniques, however, are limited in their use for more extensive production systems, for example, sheep grazing in upland areas.

#### **1.4.1.6 Laser Methane detector (LMD)**

The SA3CO6A Laser Methane detector (LMD) (Tokyo Gas Engineering Ltd., 2006) was originally developed for industrial detection of methane gas leaks and build-up using infrared spectroscopy. It is a hand-held detector, which uses measurements of gas column density for gases containing methane (Chagunda *et al.*, 2013). The LMD uses infrared absorption spectroscopy, in which a semiconductor laser is used as a collimated excitation source. The second harmonic detection of wavelength modulation spectroscopy is used to provide methane concentration measurements (Iseki and Miyaji, 2003 in Chagunda *et al.*, 2013). The LMD can detect methane concentrations up to 10,000 ppm-m, with a range of 150m (Chagunda *et al.*, 2013).

The potential of the LMD to estimate ruminant livestock methane emissions has been investigated by Chagunda *et al.* (2009; 2011). Point measurements taken from the nostrils of the animal for 15-25 seconds were found to have high variability (Chagunda *et al.*, 2009). However, Chagunda & Yan (2011) found a significant positive relationship between 12-16 hour LMD measurements and methane chamber measurements, when the LMD was placed inside methane chambers with dairy cows with the laser beam crossing the chamber outlet. This demonstrates that the LMD is capable of accurate

measurements of methane concentration. However, using the LMD in this way does not simplify methane emissions estimates, as the chamber outlet would not be a suitable point source of methane in an on-farm situation. Although this study demonstrated the potential of the LMD to measure methane, the method was not appropriate for use as a proxy. Chagunda *et al.* (2013) suggest that although there are differences between LMD and chamber data, the ranking of animals in terms of their methane emission, is similar between the two methods. Therefore, the LMD could be used to provide "useful decision support information through scan sampling" of cattle, thereby contributing to potential methane mitigation systems. Furthermore, Ricci *et al.* (2013) found that a model obtained using LMD data from steers and ewes could successfully predict methane as measured in methane chambers, particularly when dry matter intake and body weight were included in the model.

According to Chagunda *et al.* (2013), the advantages of LMD use include high molecular selectivity; methane can be detected within a mixture of gases, with high specificity (94%) and sensitivity (79%). The technique is also non-invasive and does not require direct animal contact, meaning that animals need not be disturbed, which may have an impact on methane measurements. Measurements are taken in real-time, with a frequency of one measurement per second; this enables small changes in methane concentration to be measured. The fact that the LMD is hand-held and portable is another advantage in terms of allowing methane measurements at the on-farm scale. Chagunda *et al.* (2013) identify several challenges in using the LMD: there are constant fluctuations in the methane concentrations measured, which makes it difficult to know what time points or the length of measurement times to use. There

may also be effects of wind speed, relative humidity, pressure and wind direction on methane concentrations; these effects become even more relevant when considering the use of the LMD in outdoor grazing situations. Further work is required to develop and validate methods of using the LMD in on-farm situations, using methane chamber data for validation. Whilst other authors have compared data from the LMD with those from animals in chambers, they have not attempted to use the LMD to estimate the volume of methane produced. The LMD is one of the potential proxies that will be the focus of this thesis.

#### **1.4.1.7 Sampling during feeding or milking**

In cattle, the potential for taking short term methane emissions measurements while animals are being milked or during feeding has been investigated (Garnsworthy *et al.*, 2012; Hegarty, 2013).

Garnsworthy *et al.* (2012) measured methane concentrations from dairy cows during milking, using infrared methane analysers positioned in each milking station. Air was continuously sampled from feeding bins in milking chambers and entered methane analysers via polyethylene tubes. The eructation frequency and the methane released by each eructation were then used to calculate methane emission rates, which could be compared with those measured using respiratory chambers. The study concluded that the method could potentially provide an accurate and reliable means of estimating daily methane output by dairy cows. This method would, however, only be appropriate for use in dairy cows and would be impractical for use in animals that are not being milked.

Another potential means of taking short term methane measurements is the GreenFeed emission monitoring unit (C-Lock Inc., Rapid City, South Dakota, USA) (Hegarty, 2013). The unit is designed to measure methane emissions from cattle when they feed from the unit during short 3–6 minute periods (Hegarty, 2013). However, the unit is designed specifically to be accessible for cattle (Hegarty, 2013), and is therefore not suitable for sheep. Animals are also required to visit units for feed; units may therefore not be suitable for use in grazing animals.

The current techniques used for sampling methane during feeding are not practical for use in sheep, particularly in grazing situations, and both techniques require investment in expensive technologies.

#### **1.4.2 Laboratory methods**

Methane production can be affected by diet and, therefore, has the capacity to be manipulated through altering diet and dietary additives. *In vitro* methods, which simulate rumen conditions, may be used to test the methane potentials of various feeds. Hristov *et al.* (2012) question the suitability of *in vitro* methods to assess the efficacy and persistence of feed-related methane mitigation strategies as they cannot take into account adaptation of the rumen ecosystem to mitigation practices, such as the introduction of a feed supplement into the diet. However, *in vitro* analysis can provide a useful starting point in the assessment and development of mitigation practices, although *in vivo* validation of these practices may also be required.

Spectroscopy techniques may also be used to explore differences between feeds, determining whether certain feed characteristics lead to them having higher or lower methane potentials.

Other laboratory methods include analysing the faeces of animals, which could be an indicator for microbial activity, and examining certain genes and enzymes, which may provide insight into the methanogenic populations of the rumen. There have been numerous studies exploring the microbial populations of the rumen and the effects of different feeds and additives on these populations. The rumen microbial ecosystem is, however, complex and further study is required to establish the relationships between methanogenic populations of the rumen and enteric methane production *in vivo* (Morgavi *et al.*, 2010).

#### **1.4.2.1 *In vitro* gas production analysis of feeds**

Early work using *in vitro* methods to determine fermentability of ruminant feeds by measuring gas produced when feeds are incubated with rumen fluid is described by McBee (1953). Trei *et al.* (1970) used an *in vitro* gas production technique involving the displacement of water to measure gas produced. This was later adapted to using the direct displacement of a syringe plunger (Czerkawski and Breckenridge, 1975), which was the same principle used in more recent experiments using the *in vitro* gas production techniques. Menke *et al.* (1979) developed the Hohenheim gas production technique and found positive correlations between *in vitro* gas production after 24 hours and *in vivo* digestibility of feedstuffs, although they concluded that 24 hours was not a sufficient incubation time for feedstuffs with higher than average rumen retention times, such as fibrous feeds. Blümmel and Ørskov (1993) used the Hohenheim gas production technique and correlated *in vitro* gas productions with *in vivo* results along with nylon bag degradabilities. Strong correlations were found between the *in vitro* and *in vivo* measurements.

Theodorou *et al.* (1994) used a similar method to other *in vitro* gas production techniques, with the modification that fermentations were performed in gas tight serum bottles. This allowed gases to collect in the head-space of bottles during fermentation. A pressure transducer was then used to periodically measure the pressure of the built-up gases in the head-space. The pressure transducer was attached via a three-way valve to a syringe and a hypodermic needle. The needle was then inserted into the head-space of the bottles, the pressure was recorded, and the syringe was drawn out until the pressure reading was zero, meaning that the pressure of the accumulated gas was equalised. Davies *et al.* (2000) used this experiment as a basis for a similar gas production method, using a fully automated system, which was found to be a useful, less labour intensive system. The France *et al.* (1993) model was used to fit curves to the gas accumulation profiles of substrates. *In vitro* gas production may be useful as a means of testing the methane potentials of feeds in conditions that simulate those of the rumen.

Although many studies evaluate the potential of *in vitro* gas production as a means of measuring methane production potentials of ruminant feeds (Getachew *et al.*, 1998), there is a paucity of available data on the methane production potentials of different grass diets. However, *in vitro* gas production has been used to demonstrate the effects of feed additives on methane production. For example, Garcia-Gonzalez *et al.* (2008) investigated the effects of a variety of plant species on *in vitro* gas production, finding that plants such as garlic and rhubarb decreased methane production. Fievez *et al.* (2003) also demonstrated the effects of fish oil on reducing *in vitro* methane production of hay. In addition, the effects of condensed tannin content on methane

production have been assessed using *in vitro* gas production (Animut *et al.*, 2008; Frutos *et al.*, 2002; Tavendale *et al.*, 2005), as discussed in Chapter 6.

#### **1.4.2.2 Spectroscopy techniques**

The advantages of using methods based on infrared spectroscopy include low unit cost, rapid-throughput of samples and no destruction of samples (Allison *et al.*, 2009). Moss and Givens (2000) investigated the use of the gas production technique and near infrared reflectance spectroscopy (NIRS) to predict *in vivo* methane production from a range of diets. It was concluded that NIRS was a good predictor of *in vivo* methane emissions but that validation and examination of a wider range of diets was needed.

Fourier-transform infrared spectroscopy (FTIR) may also be a useful tool in the determination of potential proxies for methane production. Fourier-transform infrared spectroscopy has a better spectral resolution than NIRS, and also requires only very small amounts of sample material (Allison *et al.*, 2009). It is possible to distinguish between sheep fed on different diets using FTIR of faeces (Moorby *et al.*, 2010, Parveen *et al.*, 2008). Moorby *et al.* (2010) also found that fluorescence emission spectroscopy could be used to detect differences in diet composition from faecal samples. However, FTIR was found to give better results than fluorescence emission spectroscopy, as it was able to detect differences between sampling months, which fluorescence did not. Since diet composition is known to affect methane production (Johnson & Johnson, 1995), a method which can distinguish between diets might also be used to predict methane output. FTIR has also been used to predict levels of cell wall components, such as lignin, in plant samples (Allison *et al.*, 2009). Methane production potentials of animal feeds are related to factors such as fibre content and

digestibility. Methane production potentials of ruminant feeds, and possibly of the animals that consume them, could, therefore, be predicted using FTIR.

#### **1.4.2.3 Detection of biomarkers in faeces and blood**

Archaeol is a diether lipid (2,3-diphytanyl-O-sn-glycerol) found in the cell membrane of most known archaea, including the methanogenic archaea of the rumen (Gill *et al.*, 2010). Studies have found good correlations between the number of methanogenic archaea and the amount of archaeol in anaerobic digester sludges and sea sediments (Ohtsubo *et al.*, 1993; Sunamura *et al.*, 1999). Gill *et al.* (2010) identified faecal archaeol as a potential proxy measurement for methanogenesis in the rumen.

McCartney *et al.* (2013) found a significant relationship between faecal archaeol concentration (mg/kg DM) and enteric methane output (g/kg DM intake), though the correlation coefficient was low (0.37). The use of faecal archaeol as a proxy assumes that a) faecal archaeol is an accurate indicator of rumen methanogen numbers and b) rumen methanogen numbers can be used to indicate levels of methanogenic activity.

These assumptions may not be valid: some rumen methanogens are selectively retained in the rumen and many are digested. The microbial population in the faeces is, therefore, unlikely to be representative of the ruminal microbial population.

Different growth rates of methanogens also cause differences in their activity levels; methanogen numbers do not necessarily indicate methanogenic activity (McCartney *et al.*, 2013).

Proportions of volatile fatty acids (VFAs) produced in the rumen could be used to predict methane emissions, as energy losses associated with methane production are dependent on the proportions of VFAs produced in the rumen (Johnson and Johnson,



1995). Hegarty and Nolan (2007) discussed the possibility of estimating methane production by injecting VFAs labelled with  $^{14}\text{C}$  into the rumen of either cattle or sheep to trace the rate of ruminal VFA production by measuring the dilution over time of the labelled VFAs. However, taking rumen samples, either from stomach tubing or cannulated animals, is not practical in an on-farm situation. Volatile fatty acids can be detected in blood plasma, which would be a simpler measurement to take from animals. However, due to preferential uptake of certain types of VFA by the gut mucosa and conversion of butyrate to propionate and rapid conversion of propionate to glucose, there is little correlation between VFA proportions in rumen fluid and blood plasma (Dijkstra, 1994). Monitoring rumen VFAs is not, therefore, likely to be a practical as a means of estimating methane emissions from animals at an on-farm scale.

### **1.4.3 Modelling approaches**

#### **1.4.3.1 Empirical models**

As discussed in Section 1.2.4, current emission estimates for sheep are based on Tier 1 emissions factors (i.e. the number of sheep), but there is potential to improve the accuracy of emissions estimates, using, for example Tier 2 or Tier 3 emission factors as part of empirical models to estimate methane output from animals. Resulting models could allow the calculation of an accurate estimate of methane output using easily obtained data, such as body weight.

#### **1.4.3.2 Mechanistic models**

As discussed in Section 1.3.4, mechanistic models take into account nutrient concentration of feed and could provide more accurate estimates of ruminant methane output (Kebreab *et al.*, 2008).

Benchaar *et al.* (2001) used a modelling technique in an attempt to assess the effects of nutrition strategies on methane production, finding that mathematical modelling could be useful in evaluating the differences in rumen methanogenesis between animals fed on different diets. The model used included factors such as the ratio of forage to concentrate in the diet and the nature of any dietary forage and concentrate, as well as dry matter intake.

In developing models to predict methane emissions from dairy cows, Ramin and Huhtanen (2013) found that feed intake was the main determinant of total methane production, although feeding level, diet digestibility and dietary fat concentration all had significant impacts. The effect of dietary carbohydrate composition was, however,

relatively minor. Jiao *et al.* (2014) also successfully developed equations to predict methane output by young cattle, using body weight, feed intake and energy intake.

#### **1.4.3.3 Life cycle analysis (LCA)**

Life cycle analysis (LCA) involves modelling whole-farm or production life cycle data to analyse the contribution of an animal or group of animals to GHG emissions (Hristov *et al.*, 2013). This approach estimates the potential environmental impact on producing animal products by examining inputs relative to outputs (Beauchemin & McGeough, 2012). Life cycle analysis produces values for GHG emissions intensity, or emissions per unit of product (Beauchemin & McGeough, 2012), such as per kg product, per kg protein, or per kg of average daily intake of product in a country (DeVries & DeBoer, 2010). Feeding strategies that reduce methane emissions may not result in lower total GHG intensities due to factors such as transport of animals and products and the effect of feed on production (Williams *et al.*, 2014).

## **1.5 Opportunities and challenges for reducing ruminant methane emissions**

### **1.5.1 Introduction**

Although the focus of this work was to develop methods for measurement of methane emissions by sheep, this is a step towards creating successful strategies to reduce methane emissions by sheep and ruminant livestock in general. According to Hegarty *et al.* (2010), there is limited scope for reducing ruminant methane output without limiting feed intake or animal numbers. However, increasing the amount of product, such as meat or milk, per unit of ingested feed could provide a means of reducing the environmental impact of ruminant farming, as well as the obvious economical advantages to farmers of increasing production without increasing feed costs.

### **1.5.2 Possible interventions for reducing methane output**

There is a large body of research relating to the reduction of methane from ruminant livestock; possible reduction strategies include genetic selection of low methane-yielding animals, vaccination against rumen methanogens, dietary manipulations and feed additives (Reviewed by Iqbal *et al.*, 2008). Defaunation, or elimination of the protozoa usually present in the rumen, has also been shown to reduce methane emissions from ruminants by an average of 13% (Hegarty, 1999). However, the methods available for defaunating animals, including dietary manipulation to reduce rumen pH, administration of compounds such as copper sulphate, and use of biological agents, are not viable on a commercial scale (Hegarty, 1999). Furthermore, the consequences of altering the microbial population of the rumen, other than the effect on methane production, must be taken into account: there is conflicting

evidence as to whether defaunation has a positive or negative effect on feed intake, metabolism and weight gain in ruminants (Gebeyehu & Mekasha, 2013).

Changes to livestock management practices to increase the productivity of livestock per unit of feed intake are presented as viable options for the reduction of methane per unit of animal product by Hegarty *et al.* (2010). Suggestions include the earlier mating of ewes in flocks of sheep that are self-replacing, and selection of sheep for improved residual feed intake (Hegarty *et al.*, 2010). Gerber *et al.* (2013b) reviewed methane mitigation options, taking into account the impact on productivity. Mitigation strategies were grouped into feed supplementation options and feed management options. The feed supplementation options that have been shown to be most effective are dietary lipids, nitrates and ionophores, and the feed management options with the most potential are improving forage quality, feed processing and precision feeding (Gerber *et al.*, 2013b). Hristov *et al.* (2013) recommend increasing forage digestibility and digestible forage intake as a methane mitigation strategy, which takes into account productivity. The current emphasis with regard to mitigating methane emissions is, therefore, on maximising the productivity of animals, to increase production per gram of methane emitted.

### **1.5.3 Emissions policies**

One of the challenges faced in the reduction of methane emissions by ruminants is the successful implementation of any practices that are shown to be beneficial. Hegarty *et al.* (2010) argue that there is a lack of policy to motivate farmers to reduce their emissions, with emphasis being entirely on productivity and profit. Without a means of accurately estimating methane output by sheep, or the effects of any measures taken

to reduce methane emissions by sheep at a large on-farm scale, it is difficult to introduce incentives for farmers to take measures to reduce methane emissions.

Pinares-Patiño *et al.* (2013) also highlighted the need for shorter and alternative methane emissions measurements in order to facilitate the establishment of selection lines of low-methane-producing animals, as methane output has been shown to be a heritable trait.

Proxy indicators for methane output by sheep could provide a useful tool for accurately estimating methane output by sheep at an on-farm level, as well as measuring the impact of introducing different management systems or diets on methane emissions. This is the basis of this Ph.D. project, which is introduced fully in Section 1.6.

## **1.6 Introduction to project**

### **1.6.1 Introduction**

The hypothesis upon which this project is based is that proxy indicators can be used to provide simple and accurate estimates of methane emissions by sheep that could be used at a large on-farm scale.

Sections 1.1 to 1.3 introduced the mechanisms of enteric methane production, the problems associated with methane production, and current legislation for the reporting of and reduction of GHG emissions from the agricultural sector, highlighting the importance of being able to accurately estimate methane emissions by sheep and the difficulties in doing so.

Section 1.4 detailed direct methods for measuring methane emissions from ruminant enteric fermentation, as well as several potential indirect methods, or proxies, for indicating methane output by sheep. The initial aim of this Ph.D. project was to identify proxy indicators, which could feasibly be investigated and that may provide useful estimates of enteric methane emissions from sheep. These proxies were selected using the information provided in the review of literature and are discussed in this section. Simplicity and potential to be used on a large scale were the main considerations when selecting proxies to be developed.

## **1.6.2 Proxies investigated**

Based on the review of literature, the proxies selected for development were the Laser Methane Detector (LMD), *in vitro* gas production of plant and feed samples, and prediction of methane emissions by Fourier-transform infrared (FTIR) spectroscopy. Daily methane emissions data from sheep housed in open-circuit respiration chambers and fed a range of diets were used to validate the methods explored. Much of the methane data collected from sheep in this way was generated as part of Defra project AC0115: “Improvements to the national inventory – methane.” Some of this data was collected with the help of IBERS technicians, but I was directly involved in most of the experimental work.

### **1.6.2.1 Laser Methane Detector (LMD)**

The concept of using the LMD to measure methane emissions from ruminants is relatively new, and there was, therefore, considerable scope for developing methods for its use. The nature of using the LMD is simple and non-invasive, providing a potential means to measure methane emissions with minimal disturbance to the animal. Using the LMD to measure methane is a method that requires development: the measurements taken using the LMD are measures of concentration of methane. In order for the LMD to provide useful information regarding methane output by ruminant livestock, methods must be developed to estimate daily methane emissions from the methane concentration data, which are simple enough to use at a large, on-farm scale. The experiments carried out during this project were focused on obtaining estimates of daily methane output by individual sheep, assessing the potential of the LMD to determine methane emissions from animals and exploring methodologies to achieve methane emissions estimates.



#### **1.6.2.2 *In vitro* gas production analysis**

*In vitro* gas production is a simple laboratory technique (Theodorou *et al.*, 1994), requiring relatively little feed material, which could provide measurements for methane potential of plants when incubated with rumen fluid, simulating a rumen environment. Coupled with DM intake information, this method could be used to predict methane emissions by animals on certain feeds or feed mixtures. During this project, methane production profiles for a variety of upland plants and sheep feeds were created using *in vitro* gas production analysis. The data obtained were then used to predict methane emissions by sheep, based on the feed given and the DM intake of sheep in methane chambers.

#### **1.6.2.3 Fourier-transform infrared (FTIR) spectroscopy**

Fourier-transform infrared spectroscopy is a rapid-throughput non-destructive spectroscopy method, requiring very little sample material. It could provide useful information about the components of feed material and whether any particular components affect methane potentials of feeds *in vivo* and *in vitro*. The experiments conducted in this project aimed to use the FTIR spectra of feeds to predict daily methane emissions by sheep as measured in methane chambers and *in vitro* gas production analysis of feeds.

### **1.6.3 Aims and objectives**

The aim of this thesis was to develop and validate proxy indicators for methane output by sheep, using methods that have potential to be used at a large, on-farm scale. The general objectives were as follows:

1. To identify methods that could be developed into proxy indicators for methane output by sheep, which are simple, quick, and require relatively little sample or animal interference.
2. To develop these methods, where necessary, both in terms of practical methodology and analysis of data to provide daily methane emissions estimates for sheep based on the proxies.
3. To determine whether the methods developed are repeatable.
4. To validate the methods, using daily methane emissions data from open-circuit respiration chamber experiments.
5. To evaluate the potential for each of the proxies investigated, or combinations of the proxies, to provide a means of accurately estimating methane emissions at a large on-farm scale.

More specific aims and objectives, relating to individual proxies are discussed in the experimental chapters that follow.

## Chapter 2

The use of open-circuit respiration chambers for the measurement of daily methane emissions by sheep

## **2 The use of open-circuit respiration chambers for the measurement of daily methane emissions by sheep.**

### **2.1 Introduction**

The use of open-circuit respiration chambers, or "methane chambers", to measure methane emissions by sheep is considered to be a "gold standard" and, although subject to errors and inaccuracies, is the most accurate form of methane measurement from individual sheep currently available. Throughout the project, any methane measurements taken using potential proxies were validated against methane chamber measurements that were taken either from the same animals or diets as described in the relevant experimental chapters. The method for the use of methane chambers and the experiments that were used for this validation are described in this chapter and referred back to in other chapters. The results of the methane chamber experiments are not shown in this chapter; those that are relevant to proxies are given when necessary in subsequent chapters.

#### **2.1.1 Aims and objectives**

The aim of this chapter is to give information on the principles and methods of constructing and using methane chambers to measure daily methane emissions by individual sheep, which will act as a reference point in later chapters. The objectives are as follows:

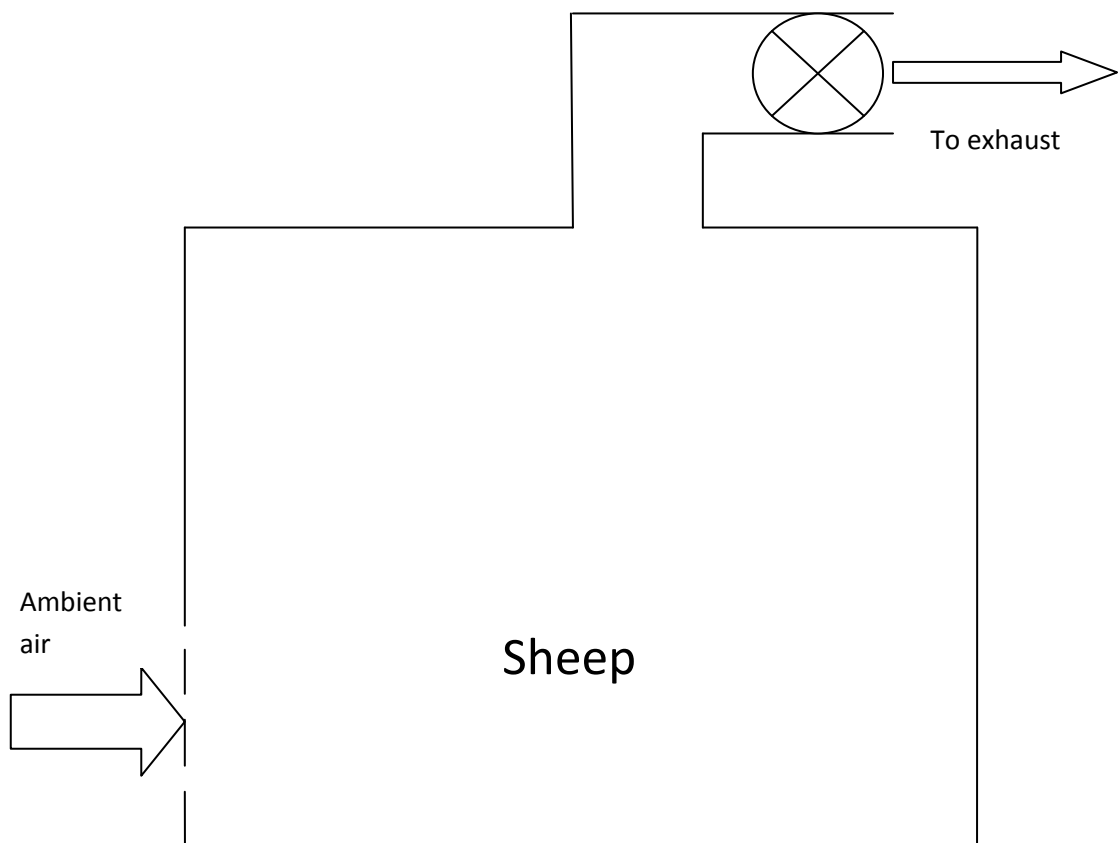
- To explain the basic principle of the use of methane chambers to measure methane emissions by sheep and to provide information about the structure of the chambers.

- To describe the practical method for the use of the methane chambers and the method for calculation of daily methane emissions (g).
- To describe specific methane chamber experiments against which proxies were validated, in order to provide a reference point for other experimental chapters.

## 2.2 General open-circuit respiration chamber method

### 2.2.1 Chamber principles

The principle of an open circuit respiration chamber (“methane chamber”) is that fresh air enters the chamber via an inlet, and air mixed with gases released by the animal exits the chamber via an outlet. The methane concentrations (ppm) sampled from the inlet and outlet gases are measured, along with the airflow through the chamber, which enables the calculation of daily methane emissions from animals inside the chambers. The general design of the chamber is shown in Figure 4.



**Figure 4: Diagrammatic representation of basic chamber principle and structure.**

### **2.2.2 Chamber structure**

The methane chambers at IBERS (Gogerddan, Aberystwyth University) were 1.8 m x 1.8 m x 1.5 m boxes (width x depth x height) constructed using a 25 mm x 25mm square tubing soft steel frame covered in clear polycarbonate sheets fixed with standard self-drilling and self-tapping screws. A 75 mm x 75 mm weld-mesh on the inside of the metal frame stopped animals damaging the polycarbonate from the inside, while the clear sheets allowed animals to see each other in neighbouring chambers. The bottom 300 mm of each side and back wall of the chambers was constructed of galvanised steel sheet to allow easy cleaning. The front of each chamber comprised two large hinged doors that had an unsealed area 300 mm high, with the same weld-mesh grid, at their bases to allow air to freely enter the chambers. The polycarbonate sheeting was sealed to the frame, using standard draft excluder tape and silicone sealant, in order to prevent as much air as possible entering or leaving the chamber in places other than an air inlet at the bottom of the chamber doors and the outlet pipe in the roof. The chambers were sited inside a sheep shed and were constructed directly on the shed's concrete floor, and the floor inside each chamber was covered with removable rubber matting for animal comfort and easy cleaning. The outlet of the chamber was in the roof, towards the back wall of the chamber, to draw air through the whole volume of the chamber.

A fan in the outflow pipe drew air through the chamber and the speed of the fan can be altered to control airflow through the chamber using a fan speed controller (ME1.1, Fläkt Woods UK Ltd, Colchester, UK). This ensures that airflow through chambers is relatively constant and that animals have a sufficient supply of fresh air. Airflow must

be controlled in order to ensure that methane concentrations remain within the detection limits of the gas analyser used. For the studies carried out for this thesis, the concentration of methane in the air analysed had to be between 0.5 ppm and 50 ppm, which were the minimum and maximum limits of analysis.

The chamber method assumes that the majority of expired gases were drawn out of the chamber via the outflow pipe. Some expired air may be lost through the inlet and around the seals of the doors, which are not completely airtight, but this was not evaluated. Instead, each chamber was calibrated to assess methane recovery as described in Section 2.2.4. Sample collection tubes, which sampled air from each chamber's outflow pipe, were connected to an 8-port single channel gas analyser (MGA-3000 multi-gas analyser, ADC Gas Analysis Ltd, Hoddeston, UK), which was set to measure methane concentration in rotation from each of the four chambers and two ambient air sampling pipes. Sampled air was drawn through an in-line desiccator comprising self-indicating silica gel ( $\text{SiO}_2$ ) desiccant in a small screw-top plastic bottle. The silica gel extracted water from the air sample to prevent interference in the gas analyser. In-line dust filters were also fitted to the sampling pipes to prevent dust contamination of the analyser. The gas analyser was set to record the methane concentration from each chamber or ambient inlet after three minutes dwell time; this allowed adjustment of the gas analyser between samples to ensure that the previous sample taken into the analyser did not have an effect on the next measurement.

### **2.2.3 Airflow and environmental measurements**

In order to calculate the daily methane output from animals, the rate of airflow through the chambers is required. This was measured using mini vane anemometers



(MiniAir 6, Schiltknecht Messtechnik AG, Gossau, Switzerland) inserted into each of the outflow pipes, connected to a battery-operated 4-channel MSR145 mini data logger (MSR Electronics GmbH, Seuzach, Switzerland). Airflow measurements for each chamber were collected every 30 seconds throughout the time sheep spent in the chambers. The airflow through chambers changed slightly throughout the day, making it necessary to take these regular measurements in order to calculate an average airflow. Noticeably, the airflow changed when lights were turned on and off in the building because this affected the power supply to the chamber fans. The datalogger also recorded ambient air temperature and pressure, which are required to convert methane concentrations in ppm to volume (see Section 2.2.6). Periodically throughout experiments, approximately once per week, the data logger was connected, via a USB cable, to a computer. The airflow and environmental data were then downloaded onto the computer.

#### **2.2.4 Chamber measurement calibration factor**

Although it is assumed that all gases released from animals in the methane chambers is extracted via the outlet pipe, it is possible that some are lost via either the chamber inlet or around the doors of the chamber. Also, a major component of the calculation of methane emissions from the chamber is the airflow measurement in the outflow pipe, and it is well known that laminar flow of gases in a pipe means that airflow at the centre of that pipe is faster than airflow close to the pipe walls. To improve the accuracy of the methane measurements, it was necessary to quantify these potential errors and correct for them. As part of Defra-funded project AC0115, one of the Defra Greenhouse Gas Platform projects, representatives from the National Physical

Laboratory (NPL) visited IBERS in December 2011 in order to calibrate the chambers, i.e. check measured values against a known amount of methane released into the chamber, and create a correction factor to minimise any differences. To do this, a device that accurately released methane at a known rate of 2.19 L/hour, was used. The device was placed inside methane chambers, as a sheep would be, and set to release methane at a constant concentration and rate, which was similar to that which would be expected from a sheep. The volume of methane released into the chamber was calculated and compared with the volume of methane measured by the chamber equipment. As expected, there were small differences in methane measurement compared to methane release; the apparent chamber capture efficiency was 0.928 ( $\pm 0.115$ ) (NPL, 2013). The NPL were able to provide a calibration factor for each of the four methane chambers, which could be applied to the daily methane calculations to increase the accuracy of methane measurements by the chambers. The calibration factors for each chamber are shown in Table 1.

**Table 1: Chamber calibration factors (NPL, 2013)**

Chamber	Calibration factor	Factor uncertainty
1	1.199	$\pm 0.082$
2	1.158	$\pm 0.029$
3	1.124	$\pm 0.074$
4	1.110	$\pm 0.152$

### **2.2.5 Chamber measurements**

Since there were four calibrated methane chambers available, sheep were placed in chambers in groups of four in each experiment. Animals were kept in the chambers for a three day period. During this time, they were released from chambers twice daily to

allow the chambers to be cleaned and for fresh feed and water to be placed in chambers. Chamber events, detailing exact times at which doors were opened and animals left and entered chambers, were recorded. This was important as any methane concentration data recorded when animals were not present in chambers or when chamber doors were open was later discarded; it would not be a true representation of methane emissions from the animals. Dates and times that sheep entered and left chambers were also recorded. Methane concentration data was checked and saved twice daily to minimise any data loss due to potential problems (e.g. power cuts) with the gas analyser or the computer that was recording measurements.

The gas analyser was set up to take samples from the sample outlet tubes of each chamber in sequence, along with two ambient samples. When a new batch of animals were put in the chambers, the methane concentrations were carefully monitored at the start and the fan speeds were adjusted if necessary to ensure that the concentrations were in the analyser's measurement range. Once three days' worth of methane concentration data was recorded for each set of four sheep, the animals were released from the chambers, which were then thoroughly cleaned before the next group entered them.

### **2.2.6 Calculation of daily methane output**

Daily methane output (g/d) was calculated using the methane concentrations measured by the gas analyser and measurements of the flow of air leaving the chambers. This was done by firstly converting the airflow ( $\text{m}^3/\text{s}$ ) into flow (L/d). This was multiplied by a "true methane concentration" (ppm), which is a mean of the

chamber methane concentration measurements minus the ambient methane concentration measurements. Flow (L/d) was multiplied by true methane concentration (ppm) and the product was divided by one million (because concentration is measured in ppm) to give daily methane output (L). All methane data were calculated to standard temperature and pressure of 0°C and 101.325 kPa regardless of the actual temperatures and pressures measured during the experiments, and methane emissions were reported in grams. The molar volume (22.414 L/mol) was calculated by multiplying Avogadro's universal gas constant (8.314462) by the standard temperature and dividing the product by the standard atmospheric pressure. Daily methane output (g) was then calculated by dividing daily methane output (L) by molar mass (L/mol) and multiplying the product by the molar mass of methane (16.04246). The daily methane output was then divided by the calibration factor, as given in Section 2.2.4, for the relevant chamber in order to correct for consistent error of chamber measurements.

## **2.3 Specific methane chamber experiments**

### **2.3.1 Introduction**

Five different chamber experiments were used as comparison points to validate various proxies, which included the use of a Laser Methane detector (LMD), *in vitro* gas production of feed samples, and Fourier-transform infrared (FTIR) spectroscopy. The general methods for the use of methane chambers are described in Section 2.2. This section provides more detail on the specific experiments, including animals used and diets given.

### **2.3.2 Small scale experiment**

#### **2.3.2.1 Animals and diet**

The animals used in this experiment were four Cheviot wethers fed on an *ad libitum* diet of grass silage. Animals were adapted to the diet for a two week period in a group pen. They were then placed in individual pens for three days before entering methane chambers for a period of five days. Animal weights were recorded upon entering and leaving chambers. Feed was offered twice daily and water was constantly available.

#### **2.3.2.2 Data and samples collected**

While animals were in individual pens and methane chambers, feed offered and refused was weighed on a daily basis. Three samples of offered feed were taken each day, along with three samples of feed refused by each sheep. These samples were weighed and then placed in an oven set at 80°C for 24 hours to remove all moisture. The samples were then re-weighed to determine dry matter (DM) content. A bulked sample, collected across the duration of time in the methane chambers, of offered feed was also taken and frozen for further analysis: some of this sample was freeze-

dried and ground through a 1mm sieve, and submitted to the analytical chemistry department (IBERS, Gogerddan) to obtain data concerning the nutritional composition of the sample. A quantity of each sample was also kept for *in vitro* gas production and FTIR analysis, which are described in the relevant experimental chapters.

Methane chamber data, which included methane concentrations, airflows, temperature and pressure, were collected, as described in Section 2.2, over a period of five days; due to technical problems involving the gas analyser, measurements taken on the first day were discarded so there were four days of chamber measurements available.

### **2.3.2.3 Data analysis**

Daily methane emissions (g) were calculated for each animal, as described in Section 2.6. These data were used as a comparison to LMD, *in vitro* gas production and FTIR spectroscopy data, the details of which are given in later chapters.

## **2.3.3 Large scale experiments**

### **2.3.3.1 Animals**

Over the course of the large scale chamber experiments, 32 sheep of 4 different breeds (Welsh mountain, Scottish blackface, Welsh mule and Texel) were used. Many of these animals were used throughout the experiments, or in more than one of the experiments, though there were some changes. However, in each of the experiments, the number of sheep of each breed was eight. Animals were weighed upon entering and leaving the methane chambers.

Chamber experiments were carried out as part of the Defra AC0115 project and IBERS technicians were responsible for collecting data from these experiments. I assisted with the data collection and used the data for my own subsequent comparisons.

### **2.3.3.2 Diets and samples and data collection**

The main difference between the series of methane chamber experiments was diet. The four diets used in experiments were zero-grazed perennial ryegrass, zero-grazed permanent pasture, zero-grazed *Molinia caerulea*, and grass nuts (made from a permanent pasture sward). All sheep in each experiment received the same diet, to which they were adapted for two weeks before the experiment began in a group pen. They were then placed in individual pens for three day periods before entering methane chambers. In the three zero-grazing experiments, sheep were fed *ad libitum*, with their feed being offered twice daily and completely refreshed each morning. In the grass nuts experiment, sheep were fed according to maintenance requirements based on their body weight. Feed was offered twice daily and any refusals, though there were rarely any, were removed once per day. Water was constantly available.

In all cases, the offered and refused feed was weighed and three samples of daily offered feed were taken while sheep were in individual pens and methane chambers. Three samples of refusals from each sheep were also taken during the zero grazing experiments; this was not necessary in the grass nuts experiments as there were rarely any refusals. These samples were weighed, dried for 24 hours in an 80°C oven, and then re-weighed to determine DM content of feeds. This, along with the feed offered and refused weights, was used to calculate DM intake by each animal. Bulk samples of offered feeds were collected over the course of each run of each methane chamber

experiment (i.e. for each group of four sheep placed in methane chambers) and frozen for future analysis. Three days of chamber measurements were taken for each sheep, as described in Section 2.2.

#### **2.3.3.3 Data analysis**

Daily methane emissions from each sheep were calculated using the method described in Section 2.2.6. These data were used to compare with proxy data based on the same sheep and diets; this is detailed in the relevant chapters for the proxies investigated.

#### **2.3.4 Further analysis of feed samples**

All bulked samples of offered feeds taken during methane chamber experiments were freeze-dried and ground through a 1mm sieve. Samples were then sent to the Analytical Chemistry department (IBERS, Gogerddan), where they were analysed to give their neutral detergent fibre (NDF), acid detergent fibre (ADF), crude protein (CP, total N  $\times$  6.25) and acid detergent lignin (ADL) concentrations. Leftover samples were then available for use in laboratory techniques that could provide proxy indicators for methane output by sheep, including *in vitro* gas production analysis and analysis by FTIR spectroscopy.

### **2.4 Conclusions**

The use of open-circuit respiration chambers is currently considered the most reliable method for obtaining daily methane emissions data from individual sheep. This method was, therefore, used during this project to obtain methane emissions data that could be used as a reference point against which data from potential proxy indicators of methane emissions by sheep could be validated. This chapter details the



principles and methods used to obtain methane emissions data from the chambers, as well as providing information regarding their construction, which was completed prior to the current project.

In terms of this project, the data collected from chamber measurements are only relevant as they relate to the data obtained from potential proxies; those that are relevant are, therefore, given in the relevant experimental chapters.

## Chapter 3

Development of a method for the use of the Laser Methane detector (LMD) to estimate daily methane output by individual sheep

### **3 The development of a method for the use of the Laser Methane detector (LMD) to estimate daily methane output by individual sheep.**

#### **3.1 Introduction**

The LMD could provide a simple, non-invasive method of collecting methane emissions data from ruminants. Though the use of lasers for micrometeorological methods of estimating methane emissions has been investigated previously (Harper *et al.*, 2011; Tomkins *et al.*, 2011), the concept of using the LMD to estimate methane emissions from individual animals is relatively new (Chagunda *et al.*, 2009). There is large scope for developing methodologies with the LMD for estimating daily methane emissions by ruminants. This chapter explores methods for the use of the LMD, including different measurement time periods and ways of taking measurements. Initial trials and a small scale study, comparing the LMD data obtained with methane chamber data from the same animals on the same diets, are detailed in this chapter.

The aim of this chapter is to outline the principles upon which LMD measurements are based and to describe its general functions, along with preliminary experiments, developing methods and evaluating the potential of the LMD to act as a proxy indicator for methane output by sheep.

## **3.2 Functions of the LMD and methods for use**

### **3.2.1 LMD Principle**

The LMD used throughout experiments was the SA3CO6A LMD from Tokyo Gas Engineering Co. Ltd. (supplied by Crowcon Detection Instruments Ltd., Abingdon, UK).

This relies on infrared spectroscopy, using a semiconductor laser to measure the concentration of methane between the LMD and the source of methane. The laser beam is transmitted towards the methane source and a fraction of the diffusely reflected beam from the target point is measured by the LMD. The measurement obtained is the methane column density (ppm-m), which is the methane concentration (ppm) multiplied by the thickness of the column (m). The LMD measurements should not be affected by gases other than methane (Tokyo Gas Engineering Co. Ltd., 2006).

### **3.2.2 Use of the LMD**

The LMD was used by simply pointing it at the methane source, pulling the trigger. The trigger could be held in place with an additional button next to it. To stop measurements the trigger was released. The LMD was set to take one measurement per second for all experiments. The LMD shown in Figure 5. The display screen, also shown in Figure 5, shows the real-time column density (ppm-m) of methane between the LMD and the point source of methane. As the data is displayed on the screen, it is saved on the machine and can later be transferred to a computer via an SD card.



**Figure 5: The Laser Methane detector (LMD) used for measuring real-time methane concentrations at a distance.**

### **3.2.3 Units of measurement**

The LMD measured methane column density in units of parts per million-metres (ppm-m). This means that concentration (ppm) was measured assuming that the distance between the LMD and source of methane was one metre. Therefore, if the distance between the LMD and source of methane was either more or less than one metre, the data needed to be corrected for the distance. This was done by multiplying the LMD measurement by the actual distance (m); for example, if the LMD was 0.85 metres away from the source, the data was multiplied by 0.85. In most of the experiments using the LMD, measurements were taken from approximately one metre away from the methane source to avoid the need to correct the data.

### **3.2.4 Battery life**

Two Ni-MH rechargeable batteries (4.8V, 2700m Ah) were supplied with the LMD. Each battery life was approximately 1.5 hours and recharging each battery took

approximately 3 hours. Protocols for individual experiments were therefore adapted to allow for the battery life and recharging time. If the LMD was left to run for the duration of the battery life, it automatically saved any data taken before it turned off, preventing any data losses.

### **3.2.5 Correcting for background methane**

The LMD has a setting to offset background methane concentration, which involves briefly pointing the LMD away from methane sources whilst pressing the offset button. However, this was tried and, due to the large number of animals in the vicinity and the presence of a muck heap near the experimental facilities, it was decided to correct data after measurements were taken rather than offsetting the LMD prior to measurements. This was done by subtracting the minimum LMD measurement from all measurements within each measurement period as this was assumed to be the background methane concentration.

### **3.2.6 Calculation of daily methane emissions**

As the output of the LMD was methane concentration, assuming that the measurement distance was one metre and no adjustment for this was required, it was necessary to calculate daily methane emissions (g) from individual sheep. The calculations developed were based on those used to calculate daily methane emissions from the methane chamber results.

#### **3.2.6.1 Airflow equivalent**

The value used as an equivalent to airflow (used in methane chamber calculations, Chapter 2, Section 2.2.6) was respiratory rate, which is calculated by multiplying tidal volume (L) with breaths per minute. Tidal volumes for sheep were estimated based on

body weight (kg): it was assumed that tidal volume (L) amounted to 12ml per kilogram of body weight, as average tidal volumes range from 10 to 15ml/kg of body weight (Kohn *et al.*, 1997). Breathing rate was assumed to be constant, using 20 breaths per minute as a normal breathing rate for sheep (University of Adelaide, 2009); this was scaled up to breaths per day (28800). Respiratory rate (L/d) was calculated by multiplying tidal volume (L) by breaths per day.

### **3.2.6.2 Integration method**

The integration, or area under the curve, of the LMD measurement was calculated in order to represent both the heights and lengths of peaks. Integration was calculated using the following formula in Microsoft Excel, assuming that the time (seconds) was in column A, the methane concentration (already corrected for background methane) was in column B, and the length of the measurement period was ten minutes (600 seconds):

$$\text{Integration} = \text{SUMPRODUCT}(A3:A601-A2:A600, (B3:B601+B2:B600)/2)$$

The integration values for all measurement periods for a particular sheep for one day were added up and, assuming that the total measurement time was thirty minutes per day for each sheep, this value was multiplied by 48 to calculate a daily value. The square root of the 'daily integration' was then taken as the integration value is an area.

### **3.2.6.3 Calculation of daily methane (L)**

Daily methane (L) was then calculated by multiplying the respiratory rate (L/d) with the square root of the daily integration and dividing the product by one million, as the units of methane concentration are parts per million (ppm).

#### **3.2.6.4 Calculation of daily methane (g)**

The molar volume of an ideal gas at standard temperature and pressure (273.15K and 100kPa respectively) is 22.4L/mol. Ambient temperature and pressure were not measured during LMD measurements, and as for the calculation of methane emissions using the chambers, this value was used, along with the molar mass of methane (16.04246 g/mol), to calculate daily methane emissions. This was achieved by dividing daily methane (L/d) by molar volume and multiplying the product by the molar mass of methane. This gave the daily methane emissions (g/d) from each animal.



### **3.3 Initial trials**

In order to be useful as a proxy, the method used to take methane concentration measurements with the LMD should be as simple as possible. Therefore, the measurement periods should be as short as possible to avoid very labour intensive methods. There were also constraints on measurement periods due to battery life and battery charging (see Section 3.2.4), which must also be considered when investigating methods to be used on a large scale. The length of measurement period and the number of measurement periods per day, taking battery life into account, was, therefore, an important methodological consideration. The method of measurement (i.e. whether animals should be held still by a handler and where the LMD should be directed) was also a consideration in these initial trials.

#### **3.3.1 Aims and objectives**

Initial work was performed with an aim of finding a useable and repeatable method for using the LMD. The objectives of the initial trials were:

- To establish an optimum measurement time period for each LMD measurement, taking into account the battery life of the LMD, the number of sheep to take measurements from and the number of measurements per day.
- To determine whether holding sheep is necessary or beneficial when taking measurements with the LMD.
- To establish whether the LMD is sensitive enough to detect eructation peaks from individual sheep, a) when pointing the LMD directly at the nostrils of the sheep, and b) when pointing the LMD at a wall behind the sheep.

### **3.3.2 Methods**

#### **3.3.2.1 Animals**

The animals used were four Cheviot wethers, which were fed on an *ad libitum* diet of grass silage, with an unlimited supply of water available. Animals were kept in individual pens, inside the sheep shed at IBERS, Gogerddan.

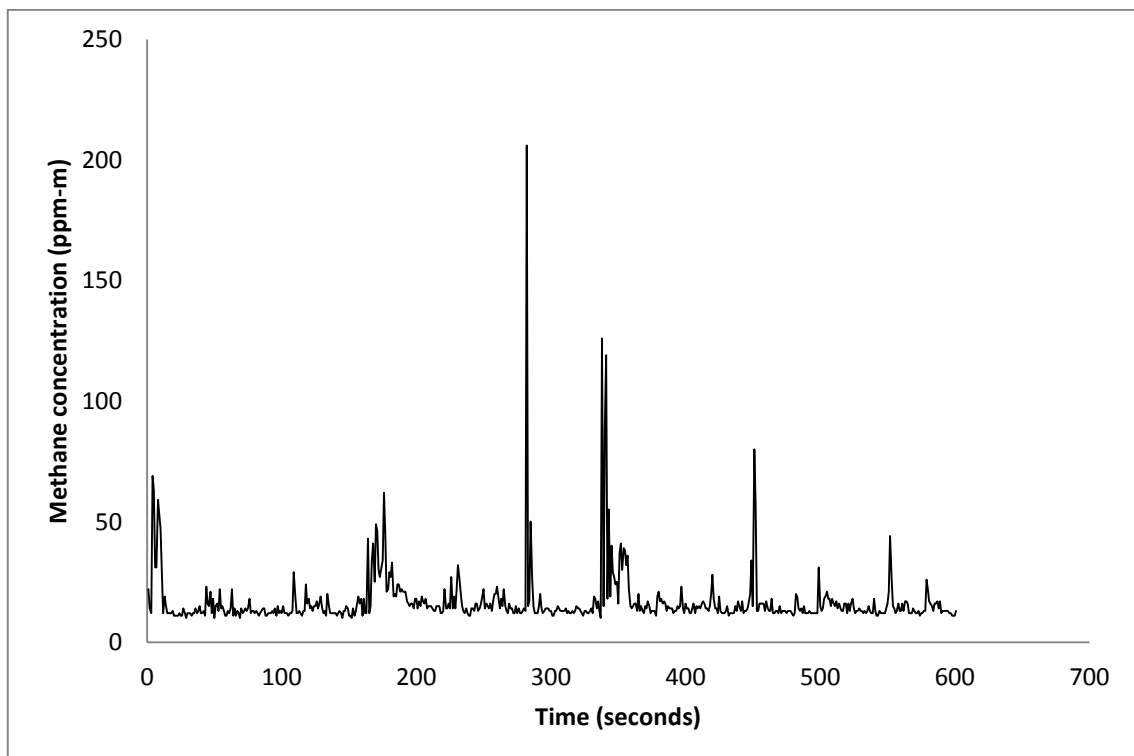
#### **3.3.2.2 Measurements taken**

There were several measurements taken from animals. The sheep stayed in the individual pens for the duration of all measurements. The standard method used was to point the LMD directly at the nostrils of sheep from approximately one metre away. When the animal was not being held, the operator moved around to maintain this distance and position of the LMD at the nostrils. Measurements were taken over different time periods (5, 10 and 15 minutes), with and without a handler holding the sheep, and with the LMD pointed above the sheep at the wall instead of directly at the nostrils.

### 3.3.3 Results

#### 3.3.3.1 Eructation peaks

Eructation peaks were clearly visible in the LMD output when the LMD was pointed directly at the nostrils of sheep (Figure 6), confirming that the LMD was sensitive enough to detect normal breathing concentrations of methane and the peaks in methane concentration associated with eructation. These eructation peaks were not observed when the LMD was pointed at the wall above the sheep. There were also unexplained peaks in methane concentration when the LMD was pointed towards certain objects. It is likely that this was due to reflectance from these objects. Pointing the LMD at the nostrils of the sheep, therefore, prevented false readings of methane, which could occur if the LMD was pointed at the wall above the sheep.



**Figure 6: Typical LMD output showing clearly visible eructation peaks**

### **3.3.3.2 Length of measurement period**

Eructation peaks were seen in the majority of measurements taken directly from the nostrils of animals. However, in the output from the five minute measurements, there were few large peaks, sometimes only one. Ten minute measurements were, therefore, considered optimum as there were always a number of clear eructation peaks in these measurements. Taking ten, rather than fifteen minute measurements, would allow more scope for taking measurements at different times of day, with regard to battery life and charging times.

### **3.3.3.3 Number of measurements per day**

There was large variation in average methane concentrations of LMD measurements taken from the same animals at different times of day. This was likely to be affected by times at which the animals were eating; eating would have an effect of increasing fermentation rates as fresh feed was consumed, as well as displacing methane in the gut, causing increased eructation. Although the animals were offered an *ad libitum* diet, they would be likely to eat most when their feed was renewed or changed. Therefore, taking some measurements directly after feeding, as well as at other times of day should be incorporated into methods. When taking ten minute measurements from four different sheep, the maximum number of measurements to be taken in a day would be three per sheep, when accounting for battery life and charging time.

### **3.3.3.4 Handling sheep**

During pilot studies to refine LMD measurement techniques, it was noticed that handling of sheep appeared to disrupt normal behaviour and reduce the number of eructation peaks, as well as complicating the method and making it more labour

intensive. The sheep were already limited in movement within their individual pens. Taking measurements whilst moving to keep the sheep in range of the LMD was simple and, as the same operator was to perform all LMD measurements in subsequent experiments, would not be affected by differences in people taking the measurements.

### **3.3.4 Conclusions**

Several conclusions were drawn from initial observations in the use of the LMD. These were used to create a protocol for a small scale experiment, testing the method suggested following the initial observations. The conclusions were as follows:

- The LMD is sensitive enough to detect both normal breathing methane concentrations and peaks in methane concentration associated with eructation, when pointed directly at the nostrils of sheep from approximately one metre away.
- Pointing the LMD at the wall or other objects can cause false peaks in methane concentration.
- The optimum measurement time, in terms of being able to take multiple measurements per day from each sheep and to include several eructation peaks, was ten minutes.
- Ten minute measurement periods would allow three measurements per day, when these measurements were taken from four sheep. These measurements should be taken at different times of day, both directly after feeding (or renewing feed) and at other time points.

- As the sheep is already restrained to an extent, by being in an individual pen, holding the sheep is not necessary and causes needless disruption to animals.

### **3.4 Small scale study comparing LMD measurements with methane chamber measurements, using four silage-fed Cheviot wethers**

#### **3.4.1 Introduction**

Having completed initial trials using the LMD, a small scale study was designed, using four Cheviot wethers, to test methods for the use of the LMD and validate them using methane chamber measurements. In this study, LMD measurements were taken while animals were in individual pens, prior to entering methane chambers, and also while animals were in methane chambers, with the LMD beam crossing the chamber outlet.

#### **3.4.2 Aims and objectives**

The aim of this experiment was to test for agreement between LMD and chamber measurements when the LMD was set up in the chamber, as well as establishing whether individual pen measurements from the LMD correlated with methane chamber measurements. The objectives were as follows:

- To determine whether there was agreement between LMD and chamber methane concentrations when the LMD was set up to take measurements across the outlet of the methane chamber for the full battery life of the LMD (approximately three hours).
- To use the conclusions from the initial observations to devise a method for taking LMD measurements from animals in individual pens.
- To compare the LMD measurements taken using this method with chamber methane measurements from the same animals fed on the same diet.
- To examine the method for calculation of daily methane emissions (g) from LMD data and to investigate whether any improvements can be made.

### **3.4.3 Materials and methods**

#### **3.4.3.1 Animals and feed**

The animals used in this trial were four Cheviot wethers (as used for initial trials), which were adapted to and fed on an *ad libitum* diet of grass silage. The feed adaptation period was two weeks. Silage was fed twice daily at specific times, which were staggered by twelve minutes between animals. This was in order to take LMD measurements at the same time point after feeding for each animal. Although the animals were fed on an *ad libitum* basis, they would almost invariably eat immediately after their feed was renewed, making the time of feeding an important consideration. Water was available constantly. Animal weights were recorded as they entered and left the chambers.

#### **3.4.3.2 DM intake and feed samples**

Silage was weighed as the animals were fed and feed refusals were also weighed. Three representative samples of each refusal, as well as three representative samples of offered feed, were taken on a daily basis, weighed, oven-dried, and re-weighed. This gave values for DM content of feed, so that DM intakes could be calculated. A bulk sample of offered feed was taken over the course of the experiment; this was freeze-dried and ground to 1mm. A sample was then sent to the analytical chemistry laboratory at IBERS, Gogerddan in order to determine NDF, ADF and WSC concentrations. This freeze-dried sample was also used as a standard sample during *in vitro* gas production experiments (Chapter 6).



### 3.4.3.3 LMD measurements in individual pens

LMD measurements were taken three times per day per sheep, for periods of ten minutes over the course of three days, while animals were in individual pens. The first measurement period was in the morning (8:30am-9:30am), forty minutes after feeding. The second was in the early afternoon (12:30pm-13:30pm), and the third was later in the afternoon (16:00pm-17:00pm), forty minutes after the afternoon feed. The method used to take LMD measurements is described in Section 3.2.2. The measurements were taken from approximately one metre away from the animal, pointing the LMD directly at the nostrils of animals (Figure 7). Animals were free to move within individual pens and the operator of the LMD moved around to keep the LMD pointing at the nostrils and to maintain the same distance between the LMD and the sheep as much as possible.



Figure 7: Method for taking LMD measurement

#### **3.4.3.4 Methane chamber measurements**

Animals were placed in methane chambers for a period of five days; the data from the first day was discarded due to problems with the gas analyser, resulting in four days of methane chamber results. The method for the use of the methane chambers is described in Chapter 2, Section 2.2.

#### **3.4.3.5 LMD measurement inside methane chambers**

During chamber measurement periods, the LMD was set up in the corner of the methane chamber, with the laser beam crossing the chamber outlet. A piece of cardboard was placed on the other side of the chamber outlet to stop the laser beam from hitting the polycarbonate wall of the methane chamber, which may have caused false methane concentration peaks due to reflectance. The distance between the LMD and the cardboard was measured. The battery was changed after approximately 1.5 hours, and two batteries were available, giving a total measurement period of three hours. The LMD was placed in each of the four methane chambers on consecutive days, so there was one three hour LMD measurement for each of the sheep.

#### **3.4.3.6 Calculations of daily methane emissions**

The LMD results were corrected for background methane concentrations using the method described in this chapter, Section 3.2.5. Daily methane emissions (g) from chamber measurements were calculated using methane concentrations from the gas analyser and flow rates through chambers, as explained in Chapter 2, Section 2.2.6. The method described in this Chapter 2, Section 2.2.6 was used to calculate daily methane emissions from the LMD measurements.

#### **3.4.3.7 Alternative calculations of daily methane emissions**

An alternative daily methane emissions estimate was also calculated. This relied on the assumption that an eructation peak could be defined in the LMD data by being more than one standard deviation (SD) greater than the mean. The LMD data was then divided into measurements considering eructation peaks and measurements considered normal breathing concentrations. The respective integrations, or areas under the curves, were then calculated as described in this Section 3.2.6. The "airflow equivalent" was then not only based on respiratory rate (L) estimates, but also on estimates of eructation volume (L/d). The calculations were performed as in Section 3.2.6, with the exception that separate daily methane emissions were calculated for "normal breathing" and for "eructations", based on the respective integrations of breathing and eructations in the data, and the daily estimates for respiratory rate and eructation volume (L/d). The main difficulty with this method was estimating eructation volume: it was assumed that the volume of a single eructation was 255ml; this was an average taken from Malbert & Million (1992). An eructation rate of once per minute was assumed and this was confirmed to be a reasonable approximation by observing eructation peaks as shown by the LMD output from various sheep. The total estimated daily eructation volume was therefore 367.2L/d.

#### **3.4.3.8 Data analysis**

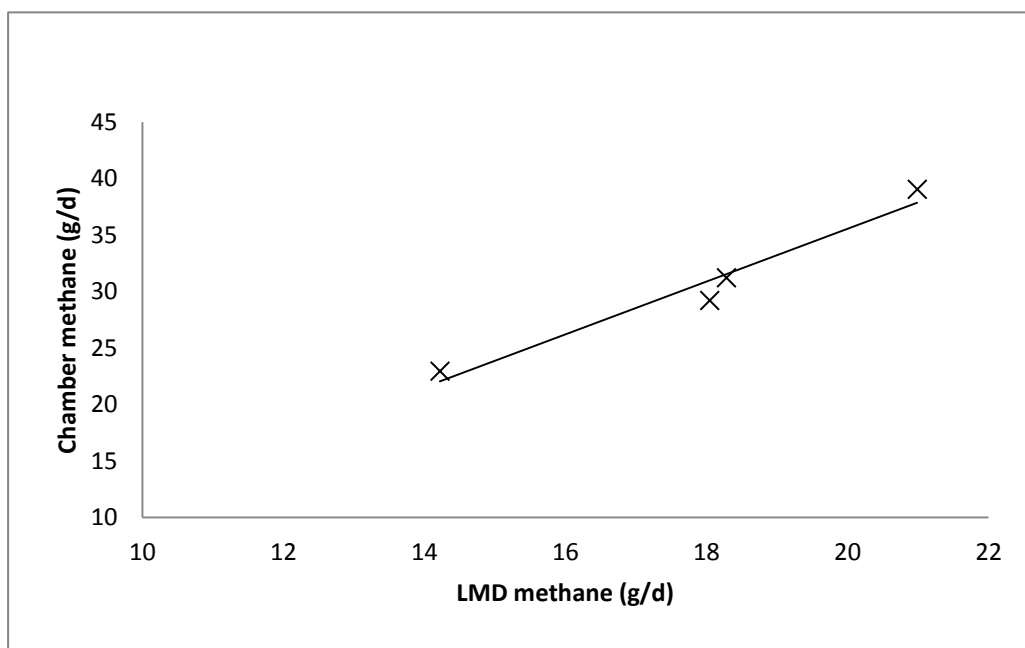
Once daily emissions were calculated, it was possible to compare daily methane emissions estimates from the LMD measurements with those from the methane chambers. This was done using simple linear regression in GenStat 16th edition (2013). Functional bisector regression would be preferred. However, a larger sample size would be required in order to conduct functional bisector regression.

The LMD measurements, which were taken inside methane chambers were compared with the chamber measurements using simple linear regression, performed in GenStat 16th edition. The methane concentrations (ppm) from the LMD were used for this comparison with the daily methane emissions (g) from the chamber measurements; the purpose of this was simply testing the sensitivity of the LMD, not attempting to estimate emissions based on these LMD measurements.

### 3.4.4 Results

#### 3.4.4.1 Comparison between daily methane emissions as measured by the LMD (in individual pens) and by methane chambers.

The LMD values appear to underestimate methane emissions as measured in methane chambers, though there is a significant correlation ( $R=0.98$ ;  $P<0.05$ ) between the LMD and chamber measurements, as shown in Figure 8. Therefore, the LMD measurements accurately predicted the ranks of animals in terms of methane emissions according to chamber measurements, though they underestimated the actual values. On average, the factor required to scale the LMD daily methane measurements to the chamber daily methane measurements was approximately 1.7, ranging from 1.62 to 1.86.



**Figure 8: Simple linear regression between chamber and LMD daily methane emissions (g/d) ( $R=0.98$ ).**

#### 3.4.4.2 Methane emissions per gram DM intake

Once again, the LMD appears to underestimate methane emissions in comparison to the methane chambers. Though the ranking of the animals in terms of their methane emissions per gram of DM intake are the same using both the chamber and the LMD measurements, in this case, the correlation between the LMD and chamber is not significant ( $P>0.05$ ). There is, however, a definite trend towards a positive correlation ( $R=0.95$ ), as  $P=0.051$ . The small sample size may be partly responsible for the lack of significance in this data. Figure 9 shows the correlation between daily methane emissions per gram of DM intake from the two types of measurement.

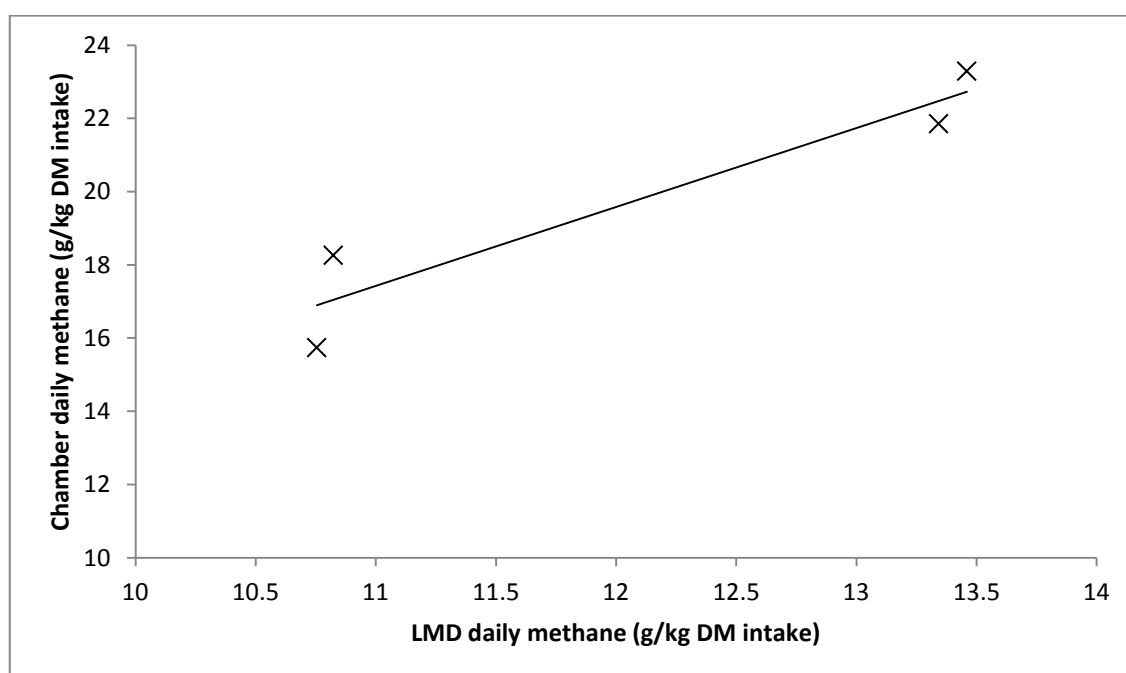
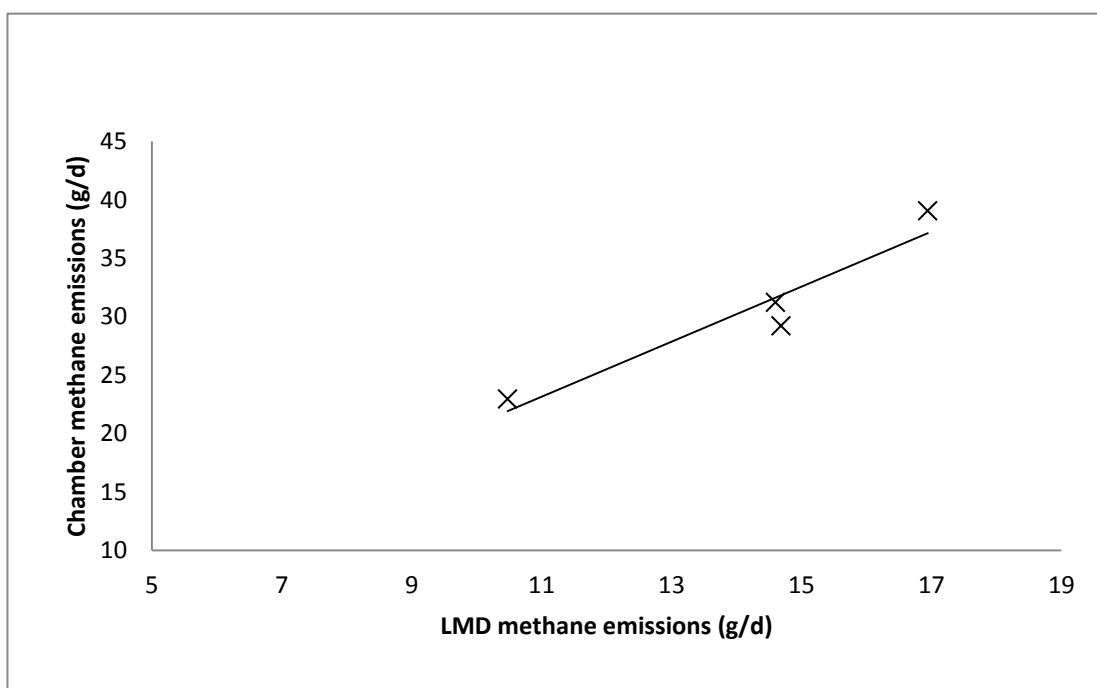


Figure 9: Simple linear regression between chamber and LMD daily methane emissions (g/kg DM intake) ( $R=0.95$ ).

#### 3.4.4.3 Daily methane emissions calculated using alternative method described in section 3.4.3.8

The daily methane emissions calculated using the alternative calculation method underestimated the daily methane emissions as measured in methane chambers.

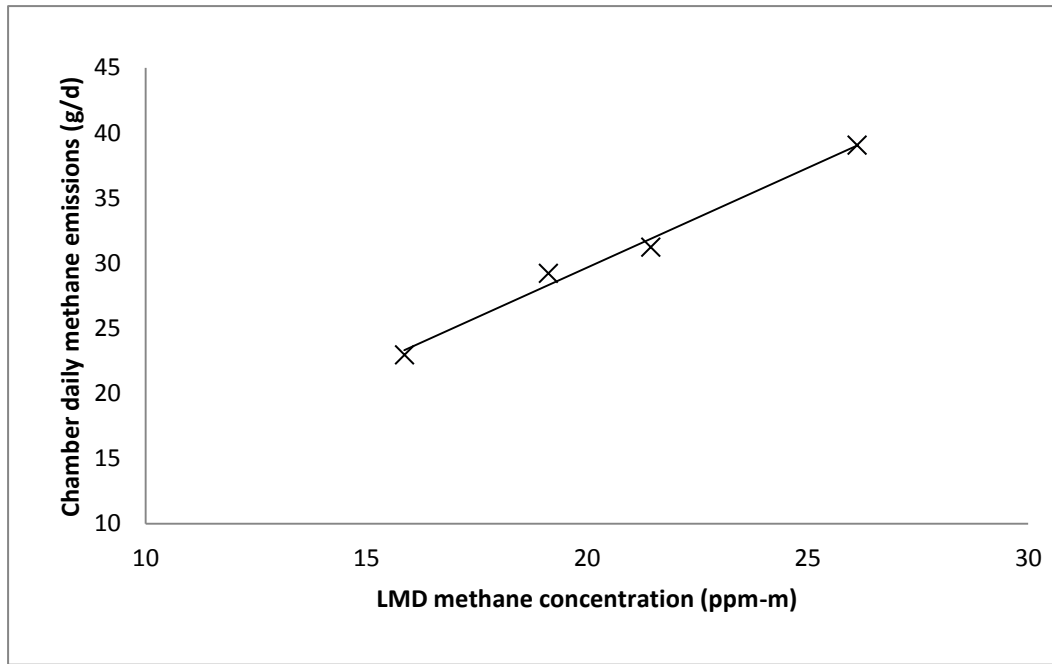
However, there is a significant positive correlation ( $R=0.96$ ,  $P<0.05$ ) between the LMD daily methane emissions calculated in this way and the daily methane emissions measured in methane chambers. This correlation is shown in Figure 10. When the correction factor of 1.7 was applied to the LMD data, the correlation was similar ( $R=0.95$ ).



**Figure 10: Simple linear regression between chamber and LMD daily methane emissions (g/d) ( $R=0.96$ ).**

#### **3.4.4.4 LMD in chamber measurements vs. daily methane emissions from chamber measurements**

There was a significant relationship ( $R=0.98$ ;  $P<0.01$ ) between daily methane emissions as measured in methane chambers and mean daily LMD methane concentrations (ppm-m). The significant positive correlation is shown in Figure 11.



**Figure 11: Correlation between LMD methane concentrations (ppm-m), as measured inside chambers and chamber daily methane emissions (g/d) ( $R=0.98$ ).**



### **3.4.5 Discussion**

#### **3.4.5.1 LMD in methane chambers, crossing the chamber outlet**

The implication from the results is that the LMD can rank sheep, in accordance with methane chamber results, in terms of their methane production. This could potentially be used to select animals in order to breed for lower methane emissions, which is a potential means of reducing ruminant methane emissions (Hegarty *et al.*, 2007). The LMD was shown to be sensitive in that there was a significant positive correlation between the LMD measurements and the daily methane emissions calculated from methane chamber data, when the LMD was placed inside the chambers. This method is not appropriate as a proxy for estimating daily methane emissions by individual animals, as it does not eliminate the need for methane chambers and complicates, rather than simplifies, methane chamber measurements. The primary use of these data was, therefore, testing for sensitivity of the LMD.

#### **3.4.5.2 Daily methane emissions as measured using the LMD when animals were in individual pens**

There were significant positive correlations ( $P < 0.05$ ) between LMD estimates of daily methane emissions, based on both methods of calculation described in Sections 3.2.6 and 3.4.3, and those measured in methane chambers. However, both methods for calculating daily methane emissions from the LMD data underestimated methane chamber values. The alternative method for calculating emissions, described in Section 3.4.3.7, more severely underestimated emissions. The problem with method arose due to the difficulty of estimating average eructation volume and number of eructations per day for use in the calculations for daily methane emissions. The results suggest that these values were underestimated. However, the volume of an eructation

is particularly difficult to estimate: this may vary with body size, diet, time of day and animal activity. There is little literature on the subject and, in a study in ewes of similar sizes ( $43\text{kg} \pm 4.3\text{kg}$ ), reported eructation volumes range from 75ml to 480 ml (Malbert and Million, 1992). Attempting to measure eructation volume would complicate an otherwise simple method. Since the alternative calculation method did not appear to improve the relationship between the LMD and chamber daily methane emissions estimates, it was concluded that, in subsequent experiments, the original calculation method (Section 3.2.6) would be used. Despite this, there may be scope to improve these calculations, incorporating eructation volume and frequency, if these data were readily available.

To address the problem of underestimation of daily methane emissions from the LMD measurements, a correction factor of 1.7 was calculated. Correction factors will be calculated using the results presented in Chapter 4 in order to establish whether the necessary correction factor is consistent and could be used in future analyses.

When the data were corrected for DM intake, the relationship between LMD and chamber daily methane emissions was not significant, though there was a definite trend towards a positive correlation ( $P=0.051$ ) and the sheep were ranked in the same order, in terms of methane output per gram of DM intake, by both the LMD and the chamber measurements. This provides further evidence that LMD measurements could be used to select sheep for breeding for lower methane emissions, which has been shown to reduce methane output in cattle (Hegarty *et al.*, 2007). Moreover, the LMD data gave plausible measurements of daily methane per kg of DM intake in light

of the relevant literature, which usually reports daily methane emissions of between 15 and 25 g/kg DM intake (Hammond *et al.*, 2013; Swainson *et al.*, 2008).

The small sample size used in this experiment makes it difficult to draw conclusions as to whether the LMD can accurately predict daily methane emissions by sheep.

However, this study does suggest that the LMD could be successfully used for this purpose, at least in terms of being able to detect differences between sheep and accurately rank them according to methane emissions. Although the calculations used to predict methane output underestimated emissions, they provided reasonable values for daily methane emissions. The underestimation could be due to some methane dissipating; the LMD was used in a comparatively open air environment, whereas, using the methane chambers, the vast majority of methane would have exited the chamber at the chamber outlet. With more available data, it could be possible to calculate correction factors to account for differences between chamber and LMD measurements, if the differences were found to be reasonably uniform.

Another issue that may arise is that there is potential for animals to re-breathe methane that has previously been eructated. This may cause methane to be measured twice, causing overestimation of methane emissions. However, as the LMD consistently underestimates methane emissions, it is likely that this effect is outweighed by the potential dissipation of the methane.

The simplicity of the method makes it potentially useable as a proxy indicator for methane output by sheep, though the method would ideally be simplified still further, in order to make it useable. Chapters 4 and 5 provide details of two larger-scaled studies using the LMD, based on the method developed.

### **3.4.5.3 Conclusions and further work**

A number of conclusions were drawn from the developmental research described in this chapter, which were used to more extensively test and validate the method for using the LMD and the calculation of daily methane emissions by individual sheep.

These were as follows:

- The LMD is sensitive enough to detect eructation peaks as well as normal breathing concentrations of methane when the laser is pointed directly at the nostrils of sheep.
- It is possible to calculate daily methane emissions by individual sheep from LMD data, which give results of a similar magnitude to calculated daily methane emissions from methane chamber data when a correction factor is included.
- When placed inside a methane chamber, the mean concentrations of methane as measured using the LMD correlate well with chamber daily methane emissions, demonstrating some level of accuracy of the LMD measurements.
- The daily methane emissions as calculated using the LMD data from animals in individual pens, accurately predicted the rank of sheep in terms of their daily methane emissions as calculated from methane chamber data.
- Further work, involving larger numbers of animals, is required to effectively validate the methods developed in this chapter. Chapters 4 and 5 provide details of two larger scale LMD experiments, which aim to validate these methods.

## Chapter 4

Validation of method for the use of the Laser Methane detector (LMD) as a potential proxy indicator for methane output by sheep

## **4 Validation of method for the use of the Laser Methane detector (LMD) as a potential proxy indicator for methane output by sheep**

### **4.1 Introduction**

Chapter 3 focuses on the development of the LMD as a proxy indicator of methane output by sheep, examining its potential to predict chamber measurements from the same sheep. Initial results suggested that the LMD could be used to accurately rank sheep in terms of their methane production. However, the initial studies were on too small a scale to establish whether the LMD method used could accurately predict methane output by sheep, although the results suggested that this was a possibility.

The aim of the work described in this chapter was to validate the method developed in Chapter 3 for the use of the LMD. In order to do this the objectives were:

- To use the method developed in Chapter 3 in large scale experiments to take LMD measurements from sheep, which were then put through methane chambers.
- To calculate daily methane emissions from the LMD output and from the chamber measurements.
- To compare daily methane emissions as measured using the two methods, using functional bisector regression to determine whether methane chamber measurements can be accurately predicted using the LMD.
- To alter methods when necessary to improve agreement between daily methane emissions measured using the LMD and methane chambers.

## **4.2 Large scale study comparing LMD and chamber estimates for animals fed on grass nuts**

### **4.2.1 Introduction**

Following the small scale study comparing the LMD and chamber measurements, the experimental protocol was adapted and expanded to test the LMD method as a means to predict methane chamber measurements on a larger scale. The aim of this experiment was to use the method used in Chapter 3 to measure and calculate daily methane emissions from the LMD on a larger scale, using 32 sheep of four different breeds, and to validate the results by comparing them with the results of a methane chamber experiment, the method for which is described in Chapter 2.

## **4.2.2 Materials and methods**

### **4.2.2.1 Animals and feed**

Thirty-two mature, barren ewes of four different breeds; Welsh mountain, Scottish blackface, Welsh mule and Texel, were used in this experiment; eight of each breed were used. The methane chamber experiment was already being carried out as part of the AC0115 project; numbers of sheep and feeds given were already established in the protocol for this project. The general method used for taking methane chamber measurements from individual sheep is explained in Chapter 2. Sheep were fed twice daily on grass nuts, according to maintenance requirements based on body weight (AFRC 1992). Animals were adapted to the diet for at least two weeks in a group pen prior to the experiment. Four animals at a time, one of each breed, were moved into individual pens for three days before entering methane chambers; four animals were used at any one time as only four methane chambers were available. Intake was recorded while sheep were in both individual pens and in the chambers; details of samples taken to obtain DM intake are given in Section 4.2.2.2. Water was constantly available for all animals throughout the experiment.

### **4.2.2.2 Feed samples**

A daily sample of offered feed was collected and bulked over the course of the three days that each set of animals spent in the chamber; this was also feed offered to animals in individual pens, which would be the next set of animals to enter the chambers. The bulked samples were freeze-dried, ground through a 1mm sieve and kept for further analysis (Section 4.2.2.3). A daily sample of offered feed was also taken, weighed, oven-dried, and weighed again to determine the DM content of feeds. Samples of refusals were not taken as, due to animals being fed to maintenance



requirements, there were generally no refusals. However, any refusals were weighed, which was necessary to determine DM intake.

#### **4.2.2.3 Sample analysis**

Freeze-dried, ground samples of offered feed, collected during each three day period while each group of sheep were in methane chambers (Section 4.2.2.2) were sent to the analytical chemistry department (IBERS, Gogerddan) in order to determine their contents including ash, crude protein (CP), neutral detergent fibre (NDF), acid detergent fibre (ADF), water soluble carbohydrate (WSC) and acid detergent lignin (ADL). Some of these parameters were later used to compare methane production by feeds containing different levels of certain components. Small samples of these offered feed samples were also used for *in vitro* gas production and FTIR analyses, which are described in Chapters 6 to 8.

#### **4.2.2.4 LMD measurements**

Measurements with the LMD were taken for the three day period while animals were in individual pens. Three ten minute measurements were taken daily from each of the four sheep in individual pens, one after each feed (morning and afternoon), and one between feeds in the early afternoon. Due to the large scale of the study, these measurement periods were not timed at exact points after feeding as in the small scale experiment (Chapter 3, Section 3.4.3.3).

#### **4.2.2.5 Methane chamber measurements**

Four animals at a time were placed in the four available methane chambers, each for a period of three days.

#### **4.2.2.6 Calculation of daily methane emissions**

The LMD results were corrected for background methane concentrations and were then converted into daily methane emissions using the integral of the measurement curve and estimated tidal volume for each sheep based on its live weight. Daily methane emissions from chamber measurements were also calculated using methane concentrations from the gas analyser and flow rates through chambers.

#### **4.2.2.7 Data analysis**

Functional bisector regression was performed in GenStat 16th edition (2013) in order to determine whether there was a significant correlation between the LMD and chamber daily methane emissions estimates and, therefore, whether the LMD measurements could predict the methane chamber measurements. This type of regression was also used to correlate methane emissions per gram of DM intake for each sheep as measured using the LMD and methane chambers. Functional bisector regression in GenStat 16th edition (2013) was also used to correlate DM intake with both LMD measurements and chamber measurements, as strong relationships have been demonstrated between DM intake and methane emissions from ruminants as described in Chapter 1, Section 1.4.1.1.

Repeated measures analysis of variance (ANOVA), using a treatment structure of Sheep with a blocking structure of Breed, was performed in GenStat 16th edition (2013) was used to compare mean methane concentrations as measured using the LMD at different times and on different days, to determine whether there were significant differences between these measurements. Repeated measures ANOVA was also used to test for significant differences between the mean methane

concentrations in the first five minutes and second five minutes for each of the ten minute measurement periods. This was in order to establish whether the measurement period could be reduced and whether the number of daily measurements and the numbers of measurement days were appropriate or should be altered for subsequent experiments; the fewer the number of measurements required, and the shorter the measurement time, the simpler and, therefore, more useful the method as a potential means of estimating daily methane output by sheep.

## 4.2.3 Results

### 4.2.3.1 Calculated daily methane emissions and DM intakes

Table 2 shows the calculated methane emissions for each sheep of each breed using the LMD and chamber measurements, along with respective DM intakes and daily methane emissions per gram of DM intake for each of the two measurement methods. Chamber daily methane emissions have been previously calculated and reported as part of Defra AC0115 project.

**Table 2: Methane emissions (g) and yields (g/kg DM intake) by breed as measured using LMD and Chamber data**

Breed	LMD daily methane emissions (g)	LMD DM intake (kg/d)	Chamber daily methane emissions (g)	Chamber DM intake (kg/d)	LMD methane yield (g/kg DM intake)	Chamber methane yield(g/kg DM intake)
WM	9.63	0.57	12.63	0.57	17.03	22.07
SB	12.51	0.73	18.50	0.73	17.18	25.34
M	15.45	0.84	17.30	0.82	18.29	21.08
T	16.61	0.87	19.84	0.85	19.02	23.35

Abbreviations: DM, dry matter; LMD, LaserMethane Detector; M, Mule; SB, Scottish Blackface; T, Texel; WM, Welsh Mountain.

### 4.2.3.2 Functional relationship using the bisector method

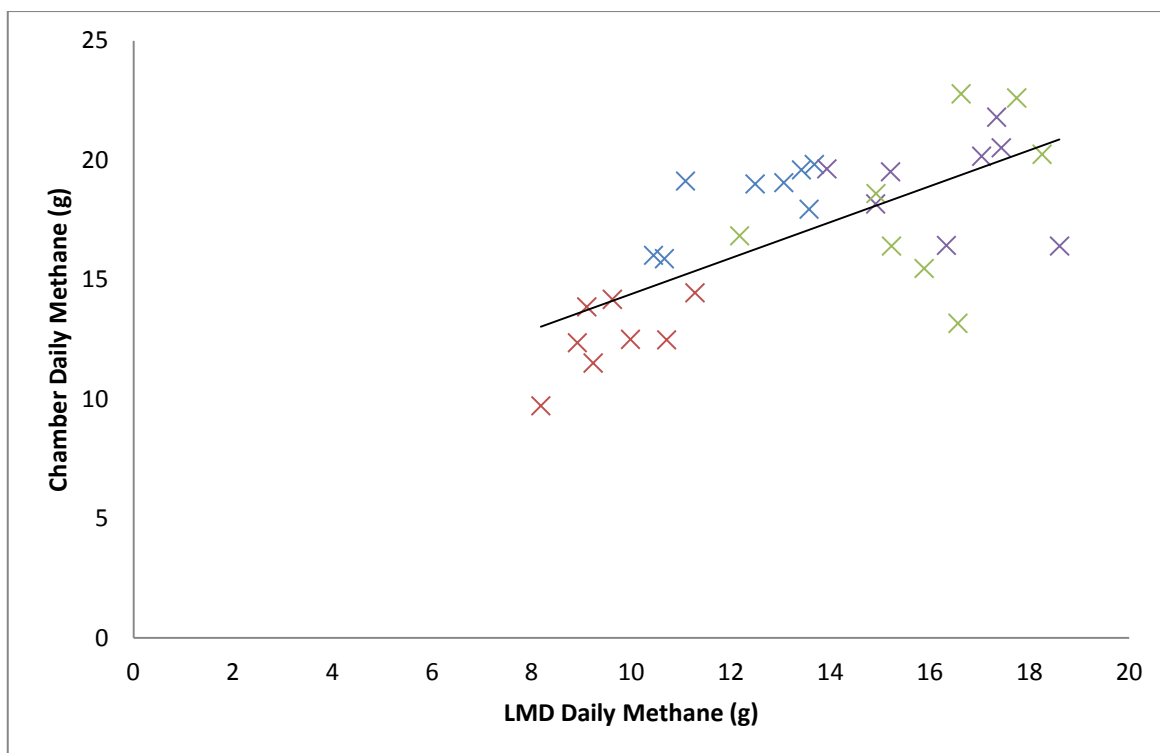
The parameters for all functional relationships are shown in Table 3. Figure 12 shows a significant positive correlation ( $R=0.70$ ,  $P<0.001$ ) between daily methane emissions estimated using the LMD and measured in methane chambers. The LMD data, therefore, successfully predicted daily methane emissions measured by methane chambers. There was no significant relationship between the LMD and chamber measurements of methane yield ( $\text{gCH}_4/\text{kg DM intake}$ ); the correlation was close to zero ( $R=0.04$ ,  $P>0.05$ ). However, there was a significant positive relationship ( $R=0.94$ ,  $P<0.001$ ) between the DM intake and the daily methane emissions estimated using the LMD. Similarly, there was a significant positive correlation ( $R=0.76$ ,  $P<0.01$ ) between DM intake and daily methane emissions measured in methane chambers. The

relationships between daily methane emissions from the sheep estimated by LMD and methane chambers and the DM intakes are shown in Figure 13 and Figure 14 respectively.

**Table 3: Parameters of functional relationships between LMD and chamber measurements of daily methane emissions (g/d) and methane yield g/g DM intake), and between LMD or chamber measurements and DM intake**

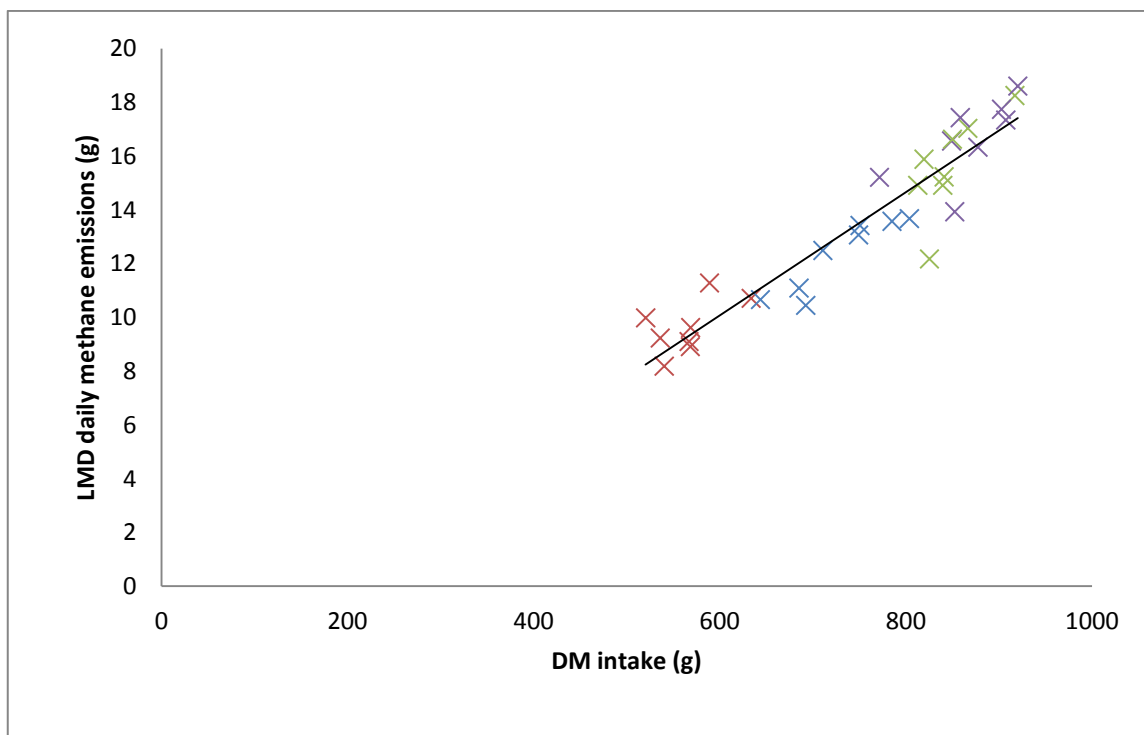
Relationship	Constant	SE	Slope	SE	Lower	SE	Upper	SE
LMD methane vs chamber methane	2.5	1.09	1.08	0.088	0.07	0.85	4.89	1.23
LMD methane/g DM intake vs chamber methane/g DM intake	0.004	0.021	1.05	1.19	-0.002	-1.60	0.052	1.40
LMD methane vs DM intake	-4.81	1.26	0.024	0.002	-7.40	0.022	-2.78	0.027
Chamber methane vs DM intake	-5.24	2.02	0.03	0.003	-10.2	0.026	-2.33	0.036

Abbreviations: DM, dry matter; LMD, laser methane detector; SE, standard error.



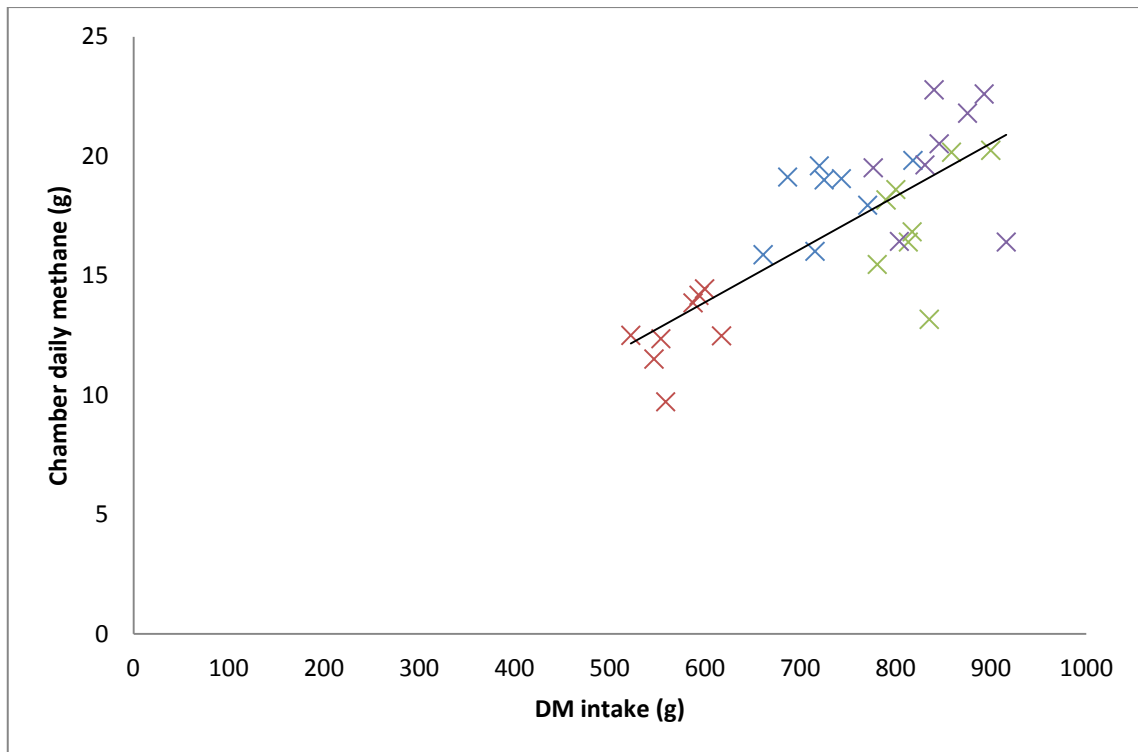
**Figure 12: Functional bisector relationship between daily methane emissions (g/d) calculated from LMD measurements and methane chamber data.**

Red=Welsh Mountain; Blue=Scottish Blackface; Green=Welsh Mule; Purple=Texel.



**Figure 13: Functional bisector relationship between LMD daily methane emissions (g/d) and DM intake (g/d).**

Red=Welsh Mountain; Blue=Scottish Blackface; Green=Welsh Mule; Purple=Texel.



**Figure 14: Functional bisector relationship between chamber daily methane emissions (g/d) and DM intake (g/d).**

Red=Welsh Mountain; Blue=Scottish Blackface; Green=Welsh Mule; Purple=Texel.

#### 4.2.3.3 Repeated measures ANOVA

Repeated measures ANOVA, using a treatment structure of Sheep with Breed as blocks, showed significant differences ( $P < 0.001$ ) between mean methane concentrations measured at different times of day (Table 4). The least significant difference (L.S.D.) at the one percent level was 1.245. When breed was used as the treatment structure, with data at different times (08:30am-9:30am, 11:30am-12:30pm, and 15:30pm-16.30pm) as repeated measures, breed did not significantly affects differences between LMD measurements at different times of day (Table 5). Repeated measures ANOVA also showed that there were no significant differences ( $P > 0.05$ ) between mean methane concentrations, as measured using the LMD, in the first and second halves of the ten minute measurement periods, or between the

overall daily methane concentrations for each sheep, shown in Table 6 and Table 7 respectively.

**Table 4: Mean LMD methane concentrations (ppm-m) at different times of day (repeated measures ANOVA)**

Measurement period	08:30am-09:30am	11:30am-12:30pm	15:30pm-16:30pm	SED	P
Mean methane concentration (ppm-m)	12.64 <sup>a</sup>	9.38 <sup>b</sup>	8.51 <sup>b</sup>	0.456	<0.001

Abbreviations: ppm-m, parts per million-metres; SED, standard error of the difference. Values in the same row with different superscripts differed significantly (P<0.01).

**Table 5: Mean LMD methane concentrations (ppm-m) at different times of day, using a treatment structure of Breed (repeated measures ANOVA)**

Time/Breed	WM	SB	M	T	SED	P
08:30-09:30	13.24	11.71	12.87	12.73	1.107	0.872
11:30-12:30	9.49	8.52	9.40	10.09		
15:30-16:30	9.38	8.06	7.96	8.62		

Abbreviations: ppm-m, parts per million-metres; SED, standard error of the difference.

**Table 6: Mean LMD methane concentrations (ppm-m) on different days (repeated measures ANOVA)**

Measurement period	Day 1	Day 2	Day 3	SED	P
Mean methane concentration (ppm-m)	9.72	10.31	10.49	0.543	0.341

Abbreviations: ppm-m, parts per million-metres; SED, standard error of the difference.

**Table 7: Mean LMD methane concentrations (ppm-m) in first and second halves of the measurement period (repeated measures ANOVA)**

Measurement period	0-5 minutes	5-10 minutes	SED	P
Mean methane concentration (ppm-m)	13.00	12.29	0.453	0.129

Abbreviations: ppm-m, parts per million-metres; SED, standard error of the difference.



## **4.2.4 Discussion and conclusions**

### **4.2.4.1 Relationship between LMD and chamber methane measurements**

There was a highly significant relationship between the LMD and methane chamber measurements for daily methane emissions, suggesting that the LMD can successfully be used to predict methane output by sheep, as measured in methane chambers.

Although a previous study (Chagunda *et al.*, 2013) has shown that the LMD has a high sensitivity (93.8%) and specificity (78.7%) when used to measure methane output by sheep, the reported relationship between LMD and chamber methane concentrations was relatively weak ( $R=0.18$ ,  $P<0.01$ ) (Chagunda *et al.*, 2013). Similar methods for LMD data collection from sheep were used in the Chagunda *et al.* (2013) publication and the current study. One difference between methods was that Chagunda *et al.* (2013) took measurements through the wall of the calorimetric methane chambers, whereas in the current study, measurements were taken directly from the nostrils of sheep, without the LMD beam passing through the polycarbonate chamber walls. Another major difference in methods was the data analysis. In the current analysis, values for daily methane emissions were calculated using methane concentrations measured using the LMD along with other measurements, such as body weight and assumptions regarding tidal volume and environmental factors. The methods used in the current analysis gave a significant positive correlation ( $P<0.01$ ) between chamber and LMD measurements (converted to daily emissions rates) with a considerably higher correlation coefficient ( $R=0.70$ ) than that shown in the Chagunda *et al.* (2013) study. Considering the simplicity of the LMD measurement method, and the number of assumptions regarding tidal volume and breathing rates, which were involved in the daily methane calculations from the LMD data, the fact that there was a significant

correlation, and a relatively high correlation coefficient, suggests that there is scope to develop the LMD method as a means of estimating methane output by sheep.

There was a tendency for the daily methane output estimates obtained using the LMD to underestimate those obtained using methane chamber data. This could be partially explained as the sheep were in a more open environment when LMD measurements were taken, as opposed to being confined to a chamber from which the majority of expired air exited via one outlet (demonstrated by the NPL (2013), see Chapter 2, Section 2.2.4). To scale the LMD daily methane measurements to the chamber daily methane measurements, an average correction factor of 1.24 was required. However, this ranged from approximately 0.80 to 1.53 in different sheep, and is not consistent with the correction factor calculated in the previous Chapter 3, which was 1.7. This suggests that the introduction of correction factors is not a sufficiently accurate method of accounting for any methane losses associated with using the LMD compared with chambers for estimating daily methane output.

#### **4.2.4.2 DM intake**

Dry matter intake was significantly correlated with both chamber and LMD daily methane emissions. This was expected as DM intake is known to be a good indicator of methane output by sheep (Molano & Clark, 2008). The correlation coefficient value was greater when correlating DM intake with the LMD emissions estimates rather than the chamber measurements. This was probably due to body weight being used as part of the calculation for daily methane emissions using LMD data: in this experiment, animals were fed according to maintenance requirements, based on their body weight. If animals were fed *ad libitum*, the relationship between daily methane

emissions estimated using the LMD and DM intake might not be expected to be as strong as body weight does not predict DM intake very accurately (Lassey *et al.*, 1997). Body weight was not used to calculate methane emissions from the chamber data, which explains why the correlation with DM intake was not as strong. However, there is still considerable variation in methane output (as measured in methane chambers and using the LMD) despite animals being fed according to body weight. If methane output was driven entirely by DM intake, a stronger and less variable relationship would be expected than the relationship between daily methane emissions (measured in methane chambers) and DM intake. The extent of variation despite feeding according to body weight suggests that a stronger relationship between DM intake and methane output cannot be achieved without a better understanding of the causes of this variation. Data obtained using the LMD could be used to further the current understanding of the variation between animals in terms of their methane output.

#### **4.2.4.3 Measurement length and frequency**

The results of the repeated measures ANOVA showed that there was no significant difference between mean methane concentrations measured using the LMD in the first and second halves of the ten minute measurement period. This implies that reducing LMD measurement periods from ten minutes to five minutes may not compromise the accuracy of the measurement. However, the sheep from which measurements were taken in this experiment were used to being handled and were reasonably relaxed with the LMD being directed at their nostrils. Although using the LMD is not an invasive procedure, it may take time for unaccustomed sheep to relax and behave normally during LMD measurements. Reducing measurement times may, therefore, not always be a sensible option.

Despite this, reducing individual measurement periods would allow more measurements to be taken per day, which would be an improvement given that the results varied significantly depending on the time of day. This variation in methane production depending on measurement time is consistent with findings from LMD measurements taken in cattle (Chagunda *et al.*, 2013). Some of the variation over the course of the day may be attributed to time of measurement as compared to the time of feeding (Chagunda *et al.*, 2013); concentrations of methane may increase after feeding due to increased enteric fermentation and displacement of gases in the rumen due to feed intake. It is, therefore, important to ensure that measurements are taken at different times of day in order that they are representative of daily methane emissions. Alternatively, if a knowledge of when measurements are taken relative to feeding was available, this could potentially be added to the model in order to account for this. The results also suggested that the LMD measurements need not be taken for three days, as there were no significant differences between mean methane concentrations on different days. Reducing the number of days on which measurements are taken would increase the simplicity of the method, making it less time-consuming and labour intensive; these attributes are essential in any proxy that is to be used at a large on-farm scale.

#### **4.2.4.4 Conclusions and recommendations**

The conclusions following this study were:

- There were significant relationships between daily methane emissions as measured using methane chambers and as estimated using the LMD.
- There was a tendency for the daily methane emissions estimated using the LMD to underestimate the daily methane emissions as measured using the methane chambers; this was consistent with the results of the small scale experiment (Chapter 3).
- Dry matter intake was a good predictor of daily methane emissions for both the chamber and LMD measurements.
- LMD measurement time could be reduced in subsequent experiments, allowing more measurement periods each day.
- The number of days required for measuring methane emissions by sheep using the LMD could be reduced from three days to two days: this would allow for group measurements to be taken on one day for each group of four sheep that are waiting to enter individual pens followed by methane chambers.

## Chapter 5

Large scale study comparing LMD and chamber estimates  
for animals fed on *Molinia caerulea*

## **5 Large scale study comparing LMD and chamber estimates for animals fed on *Molinia caerulea*.**

### **5.1 Introduction**

This experiment was a continuation of the previous studies, described in Chapter 3 and Chapter 4. Some changes to the protocol for use of the LMD were implemented based on the results of the previous experiments.

The previous study, described in Chapter 4, concluded that there was no significant difference between the mean LMD measured concentrations in the first and second five minute periods of the ten minute measurement periods. There were, however, significant differences between LMD measurements taken at different times of day. Therefore, the LMD measurement periods were made shorter and more frequent in this experiment. There were no significant differences between measurements taken on different days in the experiment described in Chapter 4, which suggested the number of measurement days could be reduced in this experiment. LMD measurements were only taken for two of the three days that animals were in individual pens. The third day could then be used to take some group measurements from animals in the group pen, which was not possible on other days due to the constraints of the battery life of the LMD.

The aim of this experiment was to use the results and conclusions of the previous experiments to adapt and improve methods for the use of the LMD to estimate methane emissions by sheep and to validate the methane emissions estimates obtained from the LMD data by comparing them with daily methane emissions calculated using methane chamber data. The objectives of this study were as follows:

- To identify the changes that should be made to the method of obtaining LMD data based on the previous experiment and to implement these changes into the method used.
- To collect LMD data while sheep were in individual pens, prior to entering methane chambers, whilst recording DM intake, and to calculate daily methane emissions from each individual sheep.
- To collect methane chamber data from sheep, and use it to calculate daily methane emissions, whilst recording DM intake.
- To compare daily methane emissions as calculated using LMD and chamber data.
- To correlate daily methane emissions, as measured using each technique with DM intake.
- To investigate whether group measurements, taken from four sheep in a group pen, could provide a reasonable estimate of daily methane emissions from animals.



## **5.2 Materials and methods**

### **5.2.1 Animals and feed**

The animals used in this experiment were 32 mature, barren ewes of four different breeds; Welsh Mountain, Scottish blackface, Welsh mule and Texel. Most were the same sheep as used in the grass nuts study (Chapter 4); a few of the animals were replaced but the numbers of each breed were consistent with the previous experiment. The animals were adapted to a diet of *Molinia caerulea* for at least two weeks before the experiment, and were zero-grazed on the same diet during the experiment. The *Molinia caerulea* was harvested as required from Pwllpeiran (an upland farm) using a Haldrup Harvester, and was kept in a large walk-in refrigerator next to the sheep shed. Feed was given on an *ad libitum* basis, offered twice daily. Fresh water was constantly available throughout the experiment.

### **5.2.2 Feed samples**

Feed offered and refusals were weighed on a daily basis for each animal, while animals were in individual pens and methane chambers. Each day, three sub-samples (approximately 100g) of feeds offered were taken, weighed, oven-dried for 24 hours and re-weighed to establish DM content of feed. A bulk sample of feed offered was also taken over each three day period during which each set of four animals were in methane chambers. These samples were freeze-dried and ground through a 1mm sieve in preparation for further analysis, as described in Section 5.2.3.

### **5.2.3 Sample analysis**

The bulked offered feed samples, which were freeze-dried and ground (Section 5.2.2), were sent to the analytical chemistry department (IBERS, Gogerddan) for analysis of

their chemical composition i.e. ash, CP, NDF, ADF, WSC and ADL. Small amounts of these samples were also reserved for *in vitro* gas production and FTIR analyses (Chapters 6 to 8).

## **5.2.4 LMD measurements**

### **5.2.4.1 Individual LMD measurements**

LMD measurements were taken, as described in Chapter 3, Section 3.2 over a period of two days, while animals were in individual pens. Measurements were taken six times per day for five minute periods. These were spaced throughout the day, both before, after and between feeds. One measurement was taken before the morning feed, one after. Another two measurements were taken between feeds, one at late morning, one at early afternoon. Another measurement was then taken before the afternoon feed, and the final measurement was taken after the afternoon feed.

### **5.2.4.2 Group LMD measurements**

LMD measurements were also taken when sheep were in group pens, with four sheep (one of each of the four breeds) in the pen at any one time. The LMD was pointed at the wall above the pen in an attempt to obtain an overall methane concentration measurement for the group of sheep. Background methane concentration was not accounted for in the group measurements; correcting the data for background methane was not possible as the measurements were representative of a group of sheep.

## **5.2.5 Chamber measurements**

Four animals at a time were placed in individual methane chambers, each for a period of three days. The method used is described in detail in Chapter 2, Section 2.2.

### **5.2.6 Calculation of daily methane emissions**

The LMD results were corrected for background methane concentrations using the method described in Chapter 3, Section 3.2.5. The LMD concentrations were then converted into daily methane emissions using the integral of the measurement curve and estimated tidal volume for each sheep, as explained in Chapter 3, Section 3.2.6. Daily methane emissions from chamber measurements were also calculated using methane concentrations from the gas analyser and flow rates through chambers, as explained in Chapter 3, Section 3.2.6. An attempt was also made to use the integration of the LMD curve, along with estimated combined respiratory rates of sheep in the group pen, to calculate a group methane emission rate. This used the same calculations as for calculating results from individual measurements, with the exception that the estimated respiratory rates of the animals in the group pen were combined to give an estimated group respiratory rate.

### **5.2.7 Data analysis**

Functional bisector regression was performed in GenStat 16th edition (2013) to determine whether there was a significant correlation between the daily methane emissions from individual sheep as measured and calculated using the LMD and methane chamber measurements. Methane emissions per gram of DM intake were calculated based on LMD and chamber measurements. Functional bisector regression was used to correlate methane emissions per gram of DM intake. DM intake was correlated with both LMD and methane chamber daily methane emissions, using the same form of regression.

Repeated measures ANOVA, using a treatment structure of sheep, was performed in GenStat 16th edition (2013) to compare LMD measurements from individual sheep taken at different times of day and on different days and to establish whether there was a significant effect of day or time of day on the daily methane estimates obtained using the LMD.

The group LMD measurements were not directly comparable to chamber measurements; they were preliminary tests to determine whether group measurements taken in the method used could provide daily methane estimates on the scale of what would be expected for a number of sheep based on what would be expected for each sheep individually. The results of the group measurements were, therefore, largely speculative.

## 5.3 Results

### 5.3.1 Calculated daily methane emissions and DM intake

Table 8 shows the mean calculated daily methane emissions for each sheep breed from both the LMD and chamber measurements, the DM intakes during the respective measurement periods for the LMD and chambers, and the calculated methane emissions per gram of DM intake. As in the previous experiments, the LMD tended to underestimate methane emissions when compared to the methane chamber results. The chamber data have been previously reported as part of the Defra AC0115 project.

**Table 8: Methane emissions (g) and yields (g/kg DM intake) by breed as measured using LMD and Chamber data**

Breed	LMD daily methane (g)	LMD DM intake (kg)	Chamber daily methane (kg)	Chamber DM intake (kg)	LMD methane yield (g/kg DM intake)	Chamber methane yield (g/kg DM intake)
WM	8.34	0.70	10.69	0.64	12.38	17.53
SB	11.51	0.94	14.29	0.82	12.62	18.86
M	15.01	0.96	14.04	0.92	16.69	15.96
T	15.74	1.01	18.87	1.05	16.15	20.03

Abbreviations: DM, dry matter; LMD, LaserMethane Detector; M, Mule; SB, Scottish Blackface; T, Texel; WM, Welsh Mountain.

### 5.3.2 Functional relationship using the bisector method

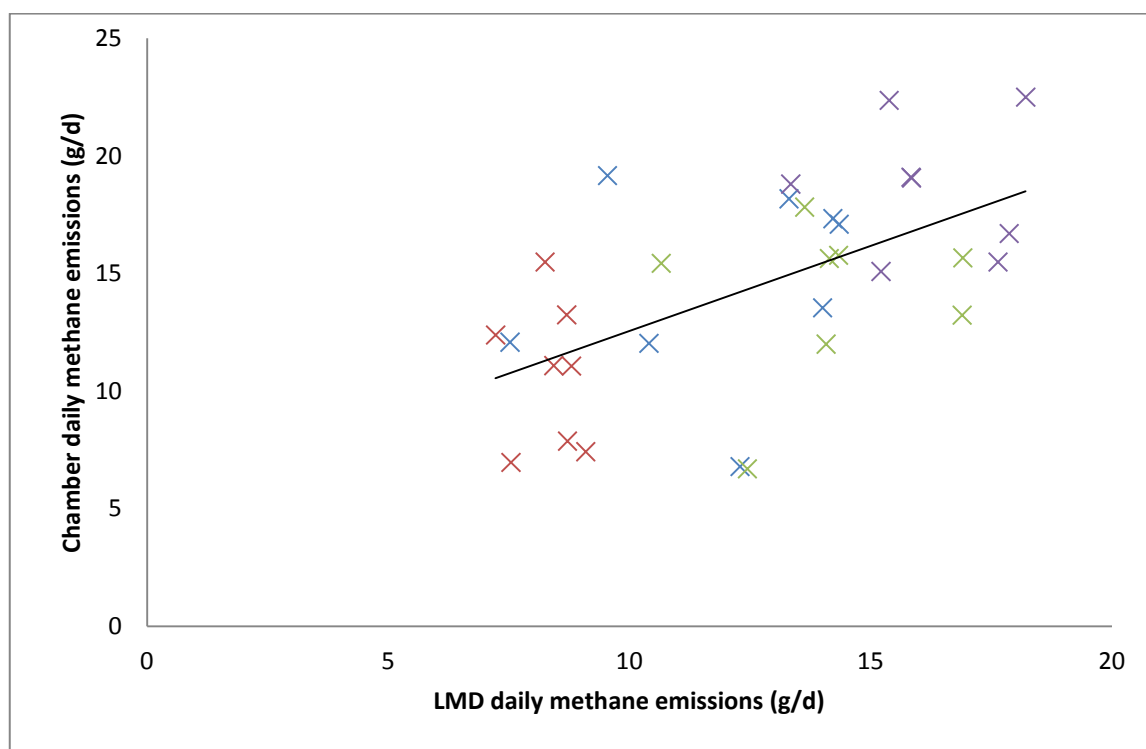
The parameters of all functional relationships are presented in Table 9. Figure 15 shows a significant positive relationship ( $R=0.57$ ,  $P<0.001$ ) between the calculated LMD and chamber daily methane emissions. Figure 16 shows a significant positive relationship ( $P<0.01$ ) between the daily LMD and chamber methane emissions per gram of DM intake. This relationship was not, however, particularly strong ( $R=0.48$ ). As in the previous experiment there were significant relationships between DM intake and daily methane emissions as calculated using LMD and chamber data ( $R=0.59$ ,

P<0.001 and R=0.40, P<0.05, respectively). These relationships are shown in Figure 17 and Figure 18.

**Table 9: Parameters of the functional relationships between LMD and chamber daily methane emissions (g/d), methane yield (g/g DM intake), and LMD and chamber measurements (g/d) vs DM intake (g)**

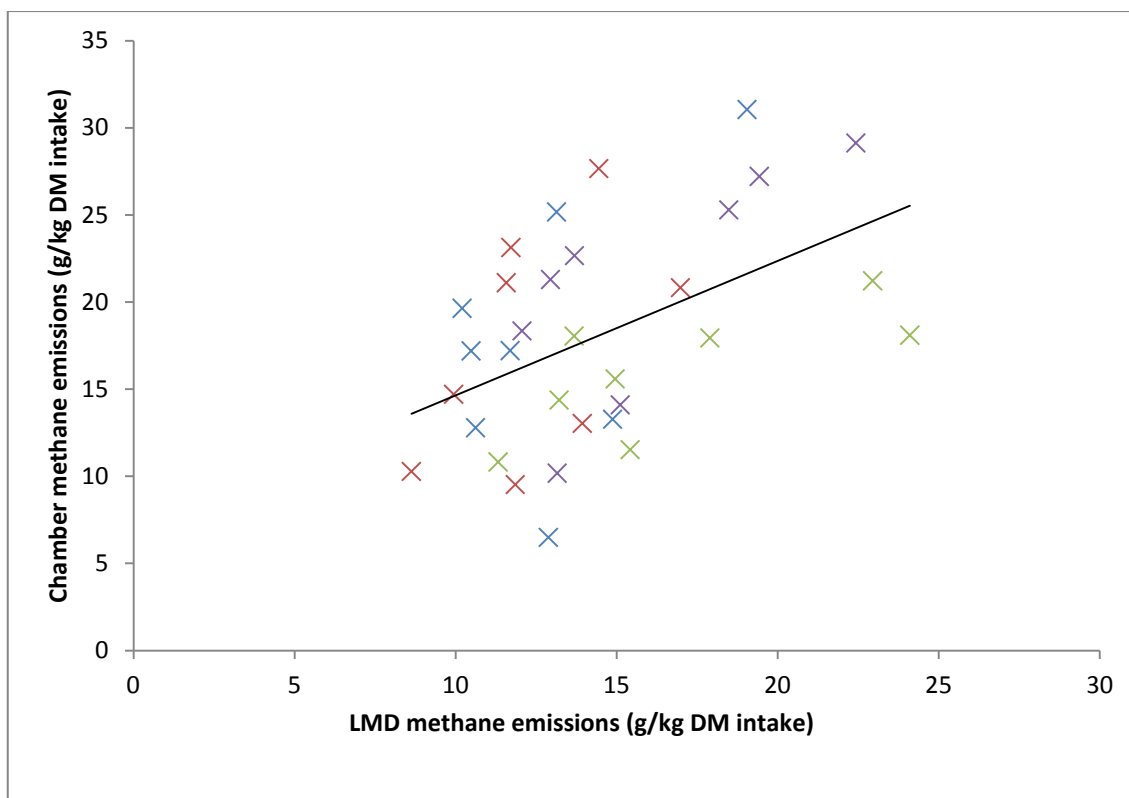
Relationship	Constant	SE	Slope	SE	Lower	SE	Upper	SE
LMD methane vs chamber methane	-0.97	2.03	1.22	0.14	-4.85	0.96	2.4	1.48
LMD methane/g DM intake vs chamber methane/g DM intake	-0.003	0.003	1.45	0.23	-0.001	1.07	0.002	1.93
LMD methane vs DM intake	-1.76	3.20	0.016	0.004	-10.16	0.013	1.18	0.027
Chamber methane vs DM intake	-3.46	17.12	0.021	0.019	-31.44	0.014	2.51	0.056

Abbreviations: DM, dry matter; LMD, laser methane detector; SE, standard error.



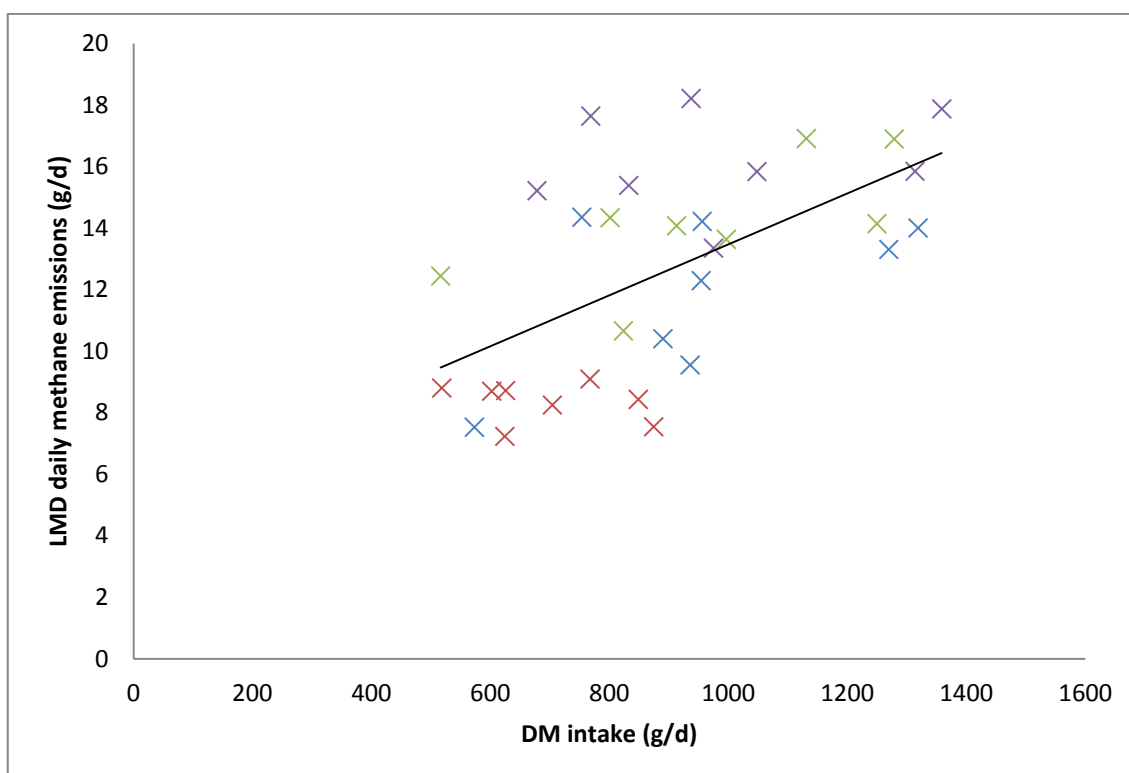
**Figure 15: Functional bisector relationship between LMD and chamber daily methane emissions (g/d)**

Red=Welsh Mountain; Blue=Scottish Blackface; Green=Welsh Mule; Purple=Texel



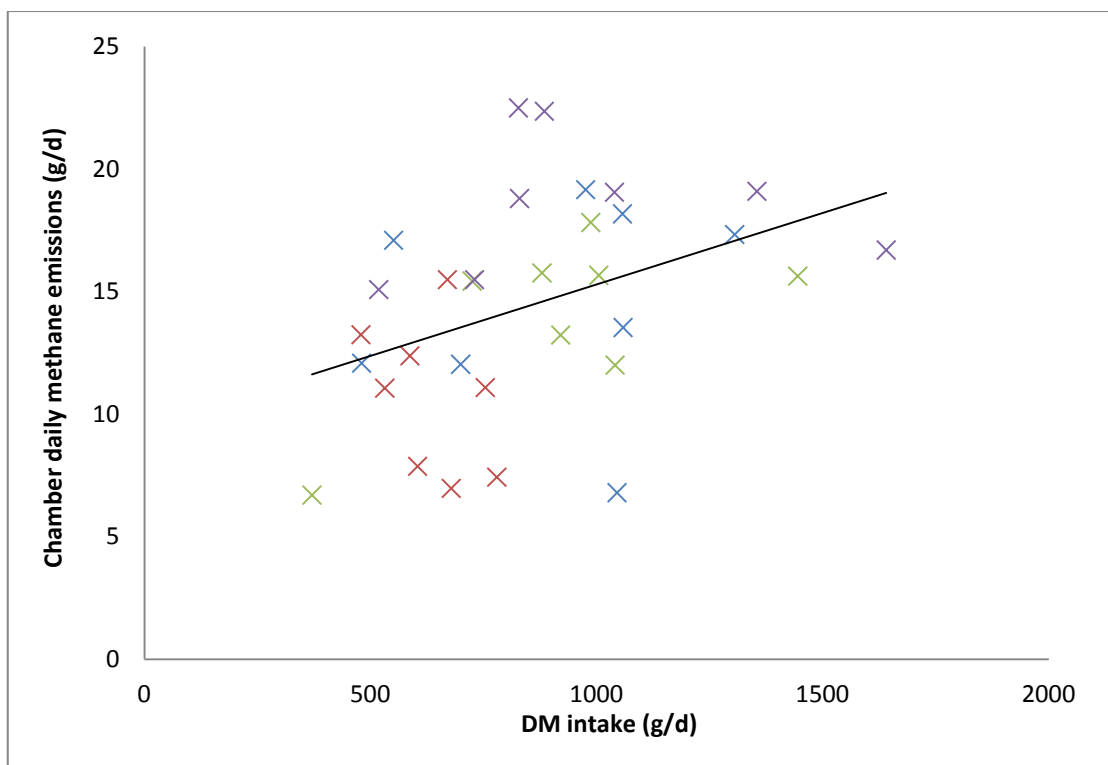
**Figure 16: Functional bisector relationship between LMD and chamber methane (g per kg DM intake)**

Red=Welsh Mountain; Blue=Scottish Blackface; Green=Welsh Mule; Purple=Texel



**Figure 17: Functional bisector relationship between LMD daily methane emissions (g/d) and DM intake (g/d)**

Red=Welsh Mountain; Blue=Scottish Blackface; Green=Welsh Mule; Purple=Texel



**Figure 18: Functional bisector relationship between chamber daily methane emissions and DM intake.**

Red=Welsh Mountain; Blue=Scottish Blackface; Green=Welsh Mule; Purple=Texel

### 5.3.3 Repeated measures ANOVA

There were no significant differences ( $P > 0.05$ ) between mean methane concentration measurements taken using the LMD on different days (Table 12). There were, however, significant differences between mean methane concentrations measured at different times of day ( $P < 0.001$ , Table 10). When sheep breed was used as the treatment structure for the repeated measures ANOVA, breed was shown to have no significant effect on differences between LMD measurements taken at different times of day (Table 11). Table 10 shows the mean methane concentrations, taken over the two day measurement period, from different times of day. The least significant difference (L.S.D.) between mean methane concentrations at a one percent level was 1.645 ppm-m. The highest methane concentrations were recorded in the second and



sixth measurement periods, which were the two measurements taken directly after feeding.

**Table 10: Mean methane concentrations (ppm-m) measured using the LMD at different times of day**

Measurement period	08:30-09:00am	10:30-11:00am	12:00-12:30pm	13:30-14:00pm	15:00-15:30pm	16:30-17:00pm	SED	P
Mean methane concentration (ppm-m)	9.54 <sup>a</sup>	11.68 <sup>b</sup>	10.13 <sup>ab</sup>	9.13 <sup>a</sup>	9.48 <sup>a</sup>	11.31 <sup>b</sup>	0.597	<0.001

Abbreviations: ppm-m, parts per million-metres; SED, standard error of the difference. Values in the same row with different superscripts differed significantly (P<0.01).

**Table 11: Mean methane concentrations (ppm-m) by breed, measured using the LMD at different times of day**

Time/Breed	WM	SB	M	T	SED	P
08:30-09:00am	9.22	8.79	8.76	11.39	1.349	0.140
10:30-11:00am	10.20	11.59	11.60	13.35		
12:00-12:30pm	9.22	10.02	10.94	10.34		
13:30-14:00pm	8.17	9.50	9.63	9.23		
15:00-15:30pm	10.70	9.11	9.18	8.95		
16:30-17:00pm	9.29	11.33	11.08	13.54		

Abbreviations: M, mule; ppm-m, parts per million-metres; SED, standard error of the difference; SB, Scottish Blackface; T, texel; WM, Welsh Mountain.

**Table 12: Mean methane concentrations (ppm-m) measured using the LMD on different days**

Measurement period	Day 1	Day 2	SED	P
Mean methane concentration (ppm-m)	10.21	10.21	0.002	0.983

Abbreviations: ppm-m, parts per million-metres; SED, standard error of the difference.

### 5.3.4 Group LMD measurements

The calculated daily methane emissions for each group of sheep and the mean daily methane emissions per sheep in each group are shown in Table 13. The figures obtained compare well with typical daily methane emissions calculated using both individual LMD measurements and methane chamber data, though the results are not

directly comparable. The results are also consistent for each group of sheep; this would be expected as the total body weights of sheep were similar for each group.

**Table 13: Calculated daily methane emissions (g/day per sheep) as measured using the LMD data obtained from groups of sheep.**

Sheep identity numbers in groups	Combined sheep weight (kg)	Daily methane emissions from group (g)	Mean daily methane emissions per sheep (g/d per sheep)
5,12,17,30	239	93	23
3,13,19,25	232	97	24
4,9,18,31	237	96	24
6,15,24,28	249	92	23
2,20,65,69	200	104	26
7,23,32,66	247	78	19
1,16,21,68	223	100	25
8,10,22,67	226	84	21

## 5.4 Discussion

### 5.4.1 Relationship between individual LMD and chamber daily methane emissions

In accordance with both the previous large scale grass nuts study (Chapter 4) and the small scale study (Chapter 3), there was a significant correlation between daily methane emissions as calculated using LMD data and methane chamber data, despite the method being slightly altered. The alterations to the method of LMD measurement, such as reducing measurement period times and increasing the frequency of measurements taken, did not appear to alter the magnitude of the correlations or the correlation coefficients much. The correlation coefficient for the relationship between LMD and chamber daily methane emissions, as calculated using functional bisector regression, in fact decreased in this experiment. However, this is not necessarily due to the alterations in methodology of LMD measurement. Despite the correlation coefficient being lower than that achieved in the grass nuts experiment, the correlation is still significantly stronger than the correlation between LMD and chamber measurements from sheep reported by Chagunda *et al.* (2013).

As in the previous experiment, the LMD tended to underestimate methane excretion by sheep. The mean factor required to correct for this underestimation was 1.18 in this experiment, which was reasonably consistent with the correction factor calculated in the previous experiment (1.24) but not with the factor calculated in the small scale experiment (1.7). However, as shown in the previous experiment, there was a wide range of required correction factors in individual sheep (0.55 to 2.01). Using the experimental methods presented in this thesis, it was not feasible to take LMD measurements at the same time as methane chamber measurements; LMD and

chamber measurements were therefore taken on different days. Although it is possible to calculate correction factors, some of the differences between LMD and chamber measurements will be due to day to day variation in methane emissions by each sheep (Pinares-Patiño *et al.*, 2013).

#### **5.4.2 Relationship between DM intake and LMD and chamber daily methane emissions**

In this experiment, there was a significant relationship between the LMD and chamber daily methane emissions per gram of DM intake, which was not the case in the grass nuts trial. However, the correlations between daily methane emissions (measured in either using the LMD or chambers) and DM intake were not as strong in this experiment as in the grass nuts trial. For the LMD data, this may be partially explained by the difference in feeding between the two experiments: in the grass nuts experiment, feed was offered according to maintenance requirements based on body weight (Alderman and Cottrill, 1993), as opposed to *ad libitum* in this trial. As body weight formed part of the calculations for daily methane emissions from the LMD data, a stronger correlation would be expected between DM intake and LMD daily methane emissions when DM intake was dependent on body weight. When DM intake is not controlled, there is significant variation in the relationship between body weight and DM intake (Lassey, 1997). Despite this, the correlation between DM intake and LMD daily methane emissions was still significant in this experiment, and, once again, this correlation was stronger than that between DM intake and daily methane emissions measured in methane chambers.

### **5.4.3 Measurement length and frequency**

As in the previous experiments, repeated measures ANOVA showed no significant differences between daily mean LMD concentrations, but significant differences between those taken at different times of day. The relatively little variation between mean daily estimates of methane measured on consecutive days suggests that taking measurements at several different times of day is an effective means of accounting for the variation over the course of the day: repeatable measurements are achieved despite significant variation in measurements taken at different times of day.

The alterations to the length and frequency of LMD measurement periods, which were made based on the results of the grass nuts experiment, did not appear to be particularly effective in improving the method, in terms of increasing the correlations or correlation coefficients. However, in this case, there was a significant correlation between LMD and chamber methane emissions measurements per gram of DM intake; there was no correlation in the grass nuts experiment.

### **5.4.4 Group LMD daily methane emissions**

Although it was not possible to directly compare the group LMD measurements to the individual chamber measurements as only a mean daily methane emission per sheep in the group could be calculated, the values obtained using the group measurements were similar to the expected daily methane emissions per sheep as measured using methane chambers. The daily methane emissions calculated per group of sheep were in a similar magnitude for each group. This would be expected as each group of sheep contained a sheep of each of the four breeds used: the total weights of each group would be similar to one another.

The implication of being able to estimate daily methane emissions based on group LMD measurements is clear: a method could potentially be developed that enables the estimation of methane emissions from large groups of animals, making it useable at a large on-farm scale. However, the data presented in this chapter for the group measurements are preliminary work and further experimentation would be required in order to determine whether this type of measurement is reliable and repeatable.

To date, the focus of published studies on the use of the LMD has focused on measurements from individual animals (Chagunda *et al.*, 2009, 2011, 2012, 2013; Chagunda, 2013; Chagunda & Yan, 2011; Ricci *et al.*, 2012). There is a lack of published data regarding the use of the LMD the estimation of methane output from groups of sheep. However, the use of open-path lasers for this purpose has been investigated with some success, although the method used overestimated methane emissions as compared to those measured in calorimetric chambers (Tomkins *et al.*, 2011).

The results of this initial experimentation suggest that further work regarding group LMD measurements should be pursued as a means of quickly and simply estimating methane output from animals, making the LMD potentially useable at a large on-farm scale.

## 5.5 General discussion of LMD experiments

The experimental data suggests that there is definite potential in the use of the LMD to estimate methane emissions by sheep. Using the calculations described in Chapter 3, Section 3.2.6, it was possible to consistently estimate values of daily methane emissions from the LMD data. In the initial small scale study and both of the larger scale experiments, these daily emissions values significantly correlated with those measured using methane chambers. The correlations were not particularly strong, and tended to have relatively low correlation coefficients. However, when the simplicity of the LMD measurement technique is taken into account, it is unsurprising that these correlations were not stronger. Despite only having thirty minutes per sheep per day of LMD data, taking measurements from sheep without them being fully restrained, and taking measurements in a barn containing other animals as sources of methane, there were still significant correlations between the LMD and chamber daily methane emissions.

In the experiments presented in Chapters 3–5 of this thesis, the LMD appeared to underestimate methane emissions as compared to the methane chambers and the mean correction factors were reasonably consistent between trials. However, there was a wide range of correction factors between individual sheep in both trials, suggesting that differences between LMD and chamber measurements may not simply be due to differences between methods. Differences between LMD and chamber measurements may be the result of a combination of factors, including differences between methods, day to day differences between methane produced by each

individual sheep (Pinares-Patiño *et al.*, 2013), and uncertainties in the method of calculating daily methane emissions from LMD data, as discussed in Section 5.5.2.2.

### **5.5.1 Methodological considerations**

#### **5.5.1.1 Repeatability of methods**

The LMD also provided repeatable methane measurements: repeated measures ANOVA showed no significant differences between mean methane concentrations of measurements taken on different days in any of the experiments. There were significant differences between mean LMD methane concentrations measured at different times of day. This was expected as methane production by sheep varies over the course of the day, higher rates of methane production being immediately after feeding (Lockyer and Champion, 2001). As there were no significant differences between daily mean methane concentrations, however, the variation throughout the day was, at least partially accounted for taking LMD measurements at different times of day. There may be scope to reduce the length and frequency of LMD measurements, which would make the method more useful as a potential proxy; this would require further investigation.

#### **5.5.1.2 Calculation of daily methane emissions**

Although other studies have examined the potential of the LMD to predict methane chamber measurements (Chagunda *et al.*, 2009, 2011, 2012, 2013; Chagunda, 2013; Chagunda & Yan, 2011; Ricci *et al.*, 2012), the work in this chapter is unique in that it shows an attempt to calculate, and successfully obtain reasonable values for daily methane emissions from sheep, using the methane concentration data from the LMD.



## **5.5.2 Potential and limitations of using the LMD to measure daily methane emissions**

### **5.5.2.1 LMD measurement method**

Though the methods used to obtain daily methane emissions estimates with the LMD in this chapter were simpler and less time-consuming than measuring methane emissions in methane chambers, taking measurements at different times of day was necessary due to the differences between measurements taken at different times of day. The LMD measurement method used also required a degree of constraint of animals, keeping them in individual pens, rather than in a grazing situation. The range of the LMD is 150m; future work could focus on adapting methods to be used in on-farm or in field situations. The initial trial of LMD measurements from groups of animals (Section 5.3.4) suggest that the LMD, or another similar device, could successfully be used on a larger scale than previously explored.

The number of measurements that could be taken daily was limited by battery life and charging times for the LMD. This is a limitation that could easily be overcome by investing in an adapter to link the LMD to mains electricity, though this would reduce the portability of the LMD. Moreover, there is potential that new machines using the same technology may be developed, which may not be subject to the same limitations in terms of battery life. For example, the use of open-path lasers is a potential means of estimating methane emissions from groups of animals in barns or fields (Tomkins *et al.*, 2011).

### **5.5.2.2 Daily methane emissions calculations**

The LMD measures the methane concentration at the nostrils of the sheep but does not provide any other information necessary to calculate daily methane emissions

from sheep. Therefore, to calculate daily methane emissions as described in Chapter 3, Section 3.2.6, it was necessary to make several assumptions to calculate respiratory rate. The LMD does not record factors such as wind speed and direction, which have been shown to have a significant impact on methane concentrations measured by the LMD (Chagunda, 2013). It may be possible to improve the measurement methods and calculations by taking these into account. If an appropriate factor for eructation volume could be developed, perhaps based in part on body weight, there may be more scope for developing an alternative, more accurate calculation method for daily methane emissions from the LMD data.

Though the calculations for daily methane emissions from the LMD data give reasonable values, which match the scale of the values given by the methane chamber data, there may be potential to improve the calculations. An attempt was made, during the initial small scale trial, to improve the method of calculating daily methane emissions from the LMD data by calculating the integrations separately for the time considered normal breathing methane concentrations and the time associated with an eructation. The emissions based on the normal breathing concentrations were then calculated using the respiratory rate as a flow rate. The emissions based on eructation peaks were calculated using an average eructation volume (Malbert & Million, 1992) and frequency. This alternative calculation method did not increase the strength of the correlations between the LMD and chamber estimates of methane emissions. In fact, using these calculations, the LMD data underestimated methane emissions compared to the chamber measurements, to a greater degree than the original calculation method. One of the difficulties in separating the normal breathing concentrations

from the eructation peaks was determining the flow rate, based on an average eructation volume. There is a lack of literature related to eructation volume, and using a constant value for eructation volume is a flawed concept: the eructation volume will vary not only between sheep, but also within individuals. However, measuring each eructation from each sheep is not possible or practical; ideally, further work could focus on the calculation of an average eructation volume based on factors such as body weight.

Despite the limitations of measurements taken using the LMD, the results presented in this thesis, particularly in Chapter 3 suggest that the LMD could be used to rank animals in terms of their methane production. As methane production appears to be a heritable trait (Hegarty *et al.*, 2007), this ranking could potentially be used for the purpose of selecting animals for breeding programmes aimed at reducing methane emissions.

## 5.6 Conclusions and Recommendations

- LMD measurements are repeatable on a day to day basis, but measurements must be taken at different times of day to ensure that the results are representative of daily methane emissions.
- There is scope to simplify methods by altering measurement times and frequency.
- The LMD can predict, to an extent, daily methane emissions, as measured in methane chambers and could provide a simple and practical method for estimating methane from sheep on a large scale.
- Daily methane emissions can be calculated, providing values that comply with the scale of those measured by methane chambers, from the LMD methane concentration measurements.
- Despite differences between daily methane emissions measured using chambers and the LMD, the ranking of sheep was generally similar between the two methods. For some purposes, ranking of sheep in terms of methane output may be sufficient; for example, for selection of low methane producing animals for breeding.
- There is potential that group measurements could be used to estimate methane output by sheep, though more work is required to investigate this possibility further.
- Calculations for daily methane emissions could be improved with more information relating to eructation volume, as well as information on wind speed and direction.

- Further work should focus on simplifying methods by reducing measurement times, investigating the use of the LMD at a greater distance from sheep, and developing calculations that rely less heavily on assumptions.

## Chapter 6

*In vitro* gas production as a proxy indicator for methane potentials of feeds

## 6 *In vitro* gas production as a proxy indicator for methane potentials of feeds

### 6.1 Introduction

#### 6.1.1 Development of the *in vitro* gas production technique

Early work using *in vitro* methods to determine fermentability of ruminant feeds by measuring gas produced when feeds are incubated with rumen fluid is described by McBee (1953). Trei *et al.* (1970) used an *in vitro* gas production technique involving the displacement of water to measure gas produced. This was later adapted to using the direct displacement of a syringe plunger (Czerkawski and Breckenridge, 1975), which was the same principle used in more recent experiments using the *in vitro* gas production techniques. Menke *et al.* (1979) developed the Hohenheim gas production technique and found positive correlations between *in vitro* gas production after 24 hours and *in vivo* digestibility of feedstuffs, although they concluded that 24 hours was not a sufficient incubation time for feedstuffs with higher than average rumen retention times, such as fibrous feeds. Blümmel and Ørskov (1993) used the Hohenheim gas production technique and correlated *in vitro* gas productions with *in vivo* results along with nylon bag degradabilities. Strong correlations were found between the *in vitro* and *in vivo* measurements.

Theodorou *et al.* (1994) used a similar method to other *in vitro* gas production techniques, with the modification that fermentations were performed in gas tight serum bottles. This allowed gases to collect in the head-space of bottles during fermentation. A pressure transducer was then used to periodically measure the pressure of the built-up gases in the head-space. The pressure transducer was

attached, via a three-way valve to a syringe and a hypodermic needle. The needle was then inserted into the head-space of bottles, the pressure was recorded, and the syringe was drawn out until the pressure reading was zero, meaning that the pressure of the accumulated gas was equalised with atmospheric pressure. The collected gas was then injected into a gas analyser, which gave percentages of methane and carbon dioxide. Therefore, the volume of methane produced could be calculated. Davies *et al.* (2000) used this experiment as a basis for a similar gas production method, using a fully automated system, which was found to be a useful, less labour intensive system. The France *et al.* (1993) model was fitted to the gas accumulation profiles of substrates. Once the model was fitted to the data, they could be compared in terms of the model parameters.

The results of the studies by Theodorou *et al.* (1994) and Davies *et al.* (2000) have shown significant differences between plant species in terms of cumulative gas production at 24, 36 and 48 hours after starting incubation (possible rumen retention times), fractional rates of degradation and total gas production potentials in the system. There were also significant correlations between the neutral detergent fibre (NDF) content of samples and the total gas production potentials in the system.

### **6.1.2 Limitations of the *in vitro* gas production technique**

The *in vitro* gas production technique requires the use of rumen fluid. The husbandry conditions, diet and timing of rumen fluid collection, may affect the results of gas production experiments, causing differences between runs using the same substrates (Rymer *et al.*, 2005). Trei *et al.* (1970) found that the volume of gas produced *in vitro*, from the same substrates, was increased when the rumen inoculum used was taken



from steers fed on grain rather than hay. Huntington *et al.* (1998), meanwhile, found no difference in gas production profiles associated with diet, and Cone *et al.* (1996) found only small dietary effects. However, according to Mauricio *et al.* (1999), rumen fluid inoculum was the greatest source of error between studies. They concluded that the rumen inoculum used should be a mixture obtained from at least two animals, offered a diet similar to the substrate. Less variation was found in rumen inoculum between samples obtained before feeding than those obtained after feeding.

Variation associated with rumen inoculum is inevitable. However, error can be reduced by using a mixture of rumen fluid from different animals fed a consistent diet, and taking samples before feeding and at the same time of day. Standard samples can also be used between studies in order to allow for corrections to be made.

Substrate preparation may also cause variation in gas production results. Whether the substrate has been oven-dried or freeze-dried and the particle size of ground substrates may affect gas production (Williams, 2000). It is, therefore, important to keep these factors consistent between substrates and studies.

### **6.1.3 Aims and objectives**

The overarching aim of the series of *in vitro* gas production experiments presented in this thesis was to establish whether the gas production profiles of plants and feeds produced using the *in vitro* gas production technique could be used to predict methane emissions from sheep fed on the same plants and feeds. The objectives of the experiments presented in this chapter were:

- To establish whether the gas production technique was sensitive enough to distinguish between methane production from a variety of upland plants, which may form part of the diet of sheep grazing in upland areas.
- To assess the effect of using a mixture of upland plant substrates with apparently contrasting methane production potentials.
- To establish whether condensed tannins present in certain upland plants (i.e. *Calluna vulgaris*, *Vaccinium myrtillis*) may be responsible for reducing the methane potentials of plants, using polyethylene glycol (PEG) to suppress the effects of tannins.
- To compare and correlate gas production results for samples with analytical chemistry data, including neutral detergent fibre (NDF), acid detergent fibre (ADF) and water soluble carbohydrate (WSC).

Validation of the gas production technique, using feed samples as predictors of *in vivo* methane output by sheep was conducted and is presented in Chapter 7, using the methods presented in this chapter.

#### **6.1.4 Samples for *in vitro* gas production**

This section outlines the samples used for gas production. All samples, including the standard samples were freeze-dried and ground to one millimetre prior to use and all samples were analysed in triplicate. In all experiments, three blank serum bottles (containing no substrate) were analysed alongside samples in order to allow correction for any methane produced without the addition of plant or feed sample substrate: blanks were expected to produce some gas due to the use of a rumen inoculum.

#### **6.1.4.1 Upland plant samples**

Fourteen species of upland plant were collected from Pwllpeiran, a 1346 ha former ADAS upland farm, approximately once per month for one year, beginning in April 2011 and ending in March 2012. These samples were collected in conjunction with John Corton, who was collecting samples for his Ph.D. in the bio-renewables research area. Plants for sampling were therefore selected based on their suitability for both projects. Table 14 shows the species of plant, the month of collection and the sample number assigned. Three samples are not present because sample plants were not found at the given site at particular times of year. In the results sections of experiments using these sample, the samples are sometimes grouped for ease of interpretation. The groups used were forbs, grasses, ferns, rushes and sedges, as shown in Table 14. In total, a large number of samples was collected (151 samples) and it was not possible to complete gas production experiments using all of these samples, particularly as all samples were analysed in triplicate. It was therefore necessary to prioritise: for example, in the first gas production experiment, the selected samples were those collected during July. This was because July would be a time of year during which all plant species would be undergoing vegetative growth and would be available for grazing. However, plant samples collected at different times of year were used in a subsequent gas production experiment in order to determine the impact of time of year on methane production potentials.

#### ***Plant sample collection methodology***

At least 100g (fresh weight) of each upland plant (if available) was collected on one day in each month. If possible, each plant species was collected from the same place

each month. Plant material, including leaves and stalks, was collected, and senescent material was collected if no or little live material was available. Fresh plant material was weighed and frozen, in preparation at freeze-drying, on return to IBERS.

#### **6.1.4.2 Standard silage sample**

To assess the impact of differences in rumen inoculum between experiments, as explained in Section 6.1.2, and to allow some correction for this, a standard sample of grass silage was used in each experiment. This sample was a feed sample collected during a small scale methane chamber experiment, in which four Cheviot wethers, fed on the grass silage, were put through methane chambers (see Chapter 2 for methods). *In vitro* gas production of this sample was, therefore, comparable with the methane chamber results from this experiment.

**Table 14: Sample number assigned to each of the upland plant samples of different species collected at different times points**

Species	Group	Month										
		2011								2012		
		Apr.	May	Jun.	Jul.	Aug.	Sept.	Oct.	Dec.	Jan.	Feb.	Mar.
<i>Calluna vulgaris</i>	Forb	1	15	29	43	57	71	85	99	113	127	141
<i>Vaccinium myrtillus</i>	Forb	2	16	30	44	58	72	86	100	114	128	142
<i>Anthoxanthum odoratum</i>	Grass	3	17	31	45	59	73	87*	101	115	129	143
<i>Deschampsia cespitosa</i>	Grass	4	18	32	46	60	74	88	102	116	130	144
<i>Molinia caerulea</i>	Grass	5	19	33	47	61	75	89	103	117	131	145
<i>Holcus lanatus</i>	Grass	6	20	34	48	62	76	90	104	118	132	146
<i>Festuca spp.</i>	Grass	7	21	35	49	63	77	91	105	119	133	147
<i>Agrostis spp.</i>	Grass	8	22	36	50	64	78	92	106	120	134	148
<i>Nardus stricta</i>	Grass	9	23	37	51	65	79	93	107*	121	135	149
<i>Juncus squarrosus</i>	Rush	10	24	38	52	66	80	94	108	122	136	150
<i>Juncus effusus</i>	Rush	11	25	39	53	67	81	95	109	123	137	151
<i>Pteridium aquilinum</i>	Fern	12*	26	40	54	68	82	96	110	124	138	152
<i>Cirsium palustre</i>	Forb	13	27	41	55	69	83	97	111	125	139	153
<i>Carex spp.</i>	Sedge	14	28	42	56	70	84	98	112	126	140	154

\* Samples not present due to plants not being found at particular times of year.

## **6.2 General methods for *in vitro* gas production**

### **6.2.1 Overview of the technique**

The *in vitro* gas production technique used in the experiment was a semi-automated method based on the method outlined by Theodorou *et al.* (1994) and Davies *et al.* (2000). This is a relatively quick and simple way of analysing samples in a simulation of rumen fermentation by incubating them with rumen fluid and measuring the amount of gas (and in particular methane) produced. Serum bottles containing samples and a digestion medium were prepared the day before inoculation with rumen fluid to allow the digestion medium to be reduced by the reducing agent and to allow the bottles to be heated to 39°C, to simulate the temperature of the rumen. The France *et al.* (1993) model was then fitted to the data, providing gas production curves and allowing comparison between plants and feeds based on the model parameters.

The preparation of gas production bottles was completed over two days before inoculation with rumen fluid. This allowed time to incubate the samples, which was necessary in order to allow for deoxygenation of the digestion medium and for increasing the temperature of the solutions to 39°C. It was important that this reduction reaction occurred as the microbes contained in rumen fluid require warm (39°C) anaerobic conditions.

### **6.2.2 Day 1**

#### **6.2.2.1 Solutions**

The following solutions were made up using distilled water boiled in a microwave oven to remove oxygen.

- Buffer solution (g/L distilled boiled water): 4g ammonium hydrogen carbonate ( $\text{NH}_4\text{HCO}_3$ ), 35g sodium hydrogen carbonate ( $\text{Na}_2\text{HCO}_3$ ).
- Macromineral solution (g/L distilled boiled water): 9.45g di-sodium hydrogen orthophosphate ( $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ ), 6.2g anhydrous potassium di-hydrogen orthophosphate ( $\text{KH}_2\text{PO}_4$ ), 0.6g magnesium sulphate 7-hydrate ( $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ ).
- Micromineral solution (g/100ml distilled boiled water): 13.2g calcium chloride 2-hydrate ( $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ ), 10.0g manganese chloride 4-hydrate ( $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ ), 1.0g cobalt chloride 6-hydrate ( $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ ), 8.0g ferric chloride 6-hydrate ( $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ ).
- Resazurin solution (g/100ml distilled boiled water): 0.1g resazurin (redox indicator).
- Reducing agent (g/100ml distilled boiled water): 0.625g cysteine HCl, 4ml 1M sodium hydroxide (NaOH).

Stock solutions of micromineral and resazurin solutions were made up and could kept refrigerated as very little was required for each gas production experiment.

#### **6.2.2.2 Sample preparation**

Samples to be used were freeze dried and ground to pass through a 1mm sieve. Approximately 1g of each sample was added to 160ml serum bottles and the weight added was recorded. Samples were analysed in triplicate so three serum bottles were used for each sample. Three bottles were left empty to act as blanks, containing no substrate. In all experiments, a standard sample of dried and ground grass silage was used to allow comparison between experiments.

### **6.2.2.3 Digestion medium preparation**

The digestion medium was prepared by mixing the prepared solutions with boiled, distilled water in the following proportions:

- 1000ml boiled distilled water.
- 0.2ml micromineral solution.
- 400ml buffer solution.
- 400ml macromineral solution.
- 2ml resazurin solution.

The volume of medium required for each gas production experiment was mixed and then CO<sub>2</sub> was passed through it for 1.5-2 hours to remove oxygen.

### **6.2.2.4 Preparation of serum bottles**

An automatic dispenser was used to add 85ml of digestion medium to each serum bottle, while gassing the bottle with CO<sub>2</sub>. A 4ml volume of reducing agent was added to each bottle immediately after the digestion medium and bungs were put in bottles as quickly as possible to reduce the amount of oxygen entering. Bottles were then sealed with aluminium seals and placed in an incubator set at 4°C. The incubator temperature was set to automatically change to 39°C early on day 2, a few hours before inoculation with rumen fluid. Bottles were ready for inoculation when the pink/purple colour of the redox indicator had faded, indicating that the solution was reduced.



## **6.2.3 Day 2**

### **6.2.3.1 Collection of rumen fluid**

An empty Dewar flask (2L volume) was taken to Trawscoed farm, approximately 20 minutes drive from IBERS, Gogerddan. The flask was filled with hot water to pre-warm it. The rumen contents were removed from three ruminally fistulated cows, which were being fed on grazed grass and grass silage. Rumen contents were squeezed through a wire sieve to separate the large solid feed particles from the rumen fluid. Rumen fluid from all animals was mixed together. The Dewar flask was emptied of water before being immediately filled with rumen fluid. The flask of rumen fluid was then transported back to IBERS, Gogerddan as quickly as possible to avoid cooling and placed in an incubator set at 39°C until needed.

### **6.2.3.2 Inoculation of serum bottles with rumen fluid**

In a fume cupboard, rumen fluid was strained through a double layer of muslin into a beaker, whilst being gassed with CO<sub>2</sub>. Fluid was mixed using a magnetic stirrer in the beaker of strained rumen fluid. A 10ml syringe fitted with a hypodermic needle was used to inject 10ml of the filtered rumen fluid through the bungs into each serum bottle. A hypodermic needle was then used to equalise the pressure, by releasing any gases, in the bottles before replacing them in the incubator at 39°C.

## **6.2.4 Data collection**

The gas analyser (ADC 5000 series, ADC Gas Analysis Ltd., Hoddesdon, UK) used to determine proportions of carbon dioxide and methane in samples was first calibrated with standard gases, one containing 80% carbon dioxide and the other containing 80% methane, the remainder being nitrogen in both cases.

The gas production bottles were removed from the incubator and placed in a water bath heated to 39°C. A three way valve was used to connect a detachable pressure transducer and LED digital readout voltmeter (Bailey & Mackey Ltd., Birmingham, UK), encased in plastic (length 200mm, depth 145mm, height 75mm; R.S. Components, Northampton, UK; constructed at IBERS Gogerddan), to a 60ml syringe and a hypodermic needle (23 gauge x 3.8cm). The needle was inserted into one bottle at a time through the bung. The syringe plunger was held in place to prevent the pressurised gas leaving the bottle while a pressure reading was taken (this was displayed by the voltmeter connected to the pressure transducer). The syringe plunger was then released and pulled out until the pressure reading reached zero, i.e. atmospheric pressure. The volume of gas removed from each bottle was recorded. The needle was withdrawn from the bottle and the gas in the syringe was injected into the gas analyser. The gas analyser provided values for percentages of carbon dioxide and methane in each sample, which were recorded. A minimum of approximately 15ml of gas was required to produce an accurate reading from the gas analyser. If there was little gas produced by samples (less than 15ml), the gas from the three triplicate samples was pooled to inject into the gas analyser. The samples were then replaced in the incubator.

Measurements were taken over a period of about 120 hours. The first measurements were taken after about 3 hours after inoculation with rumen fluid. Measurements were then taken every 3-4 hours until fermentation began to slow down and the rate of gas production decreased. The length of time between measurements was gradually increased, eventually to about 12 hours between measurements. When the

volumes of gas produced by a sample became too small to analyse, the experiment was stopped and samples were refrigerated at 4°C. Times at which measurements were taken were recorded.

### **6.2.5 Vacuum filtration of gas production products**

Crucibles for vacuum filtration were placed in a dryer for fifteen minutes to ensure that they were completely dry. They were then weighed and labelled with sample names. A rubber bung, with a hole large enough to accommodate a crucible through it, was placed in the top of a side-arm conical flask and a crucible was placed in the top of the bung. The side-arm of the flask was then connected to a water pump, creating a vacuum. A sample was then poured into the crucible. The liquid fraction of the sample was sucked into the conical flask and the solid part remained in the crucible. Once most of the liquid was removed, the crucibles were placed in a freezer. Once frozen, the samples in crucibles were freeze-dried to remove any remaining moisture. The crucibles were then weighed again to determine the weight of samples left in them. This allowed an apparent DM digestibility to be calculated; apparent digestibility calculations include the possible presence of particles and bacteria from the rumen inoculum in the filtered samples, which may have had a slight impact on the weight of filtered samples, as opposed to true digestibility, which would not include contributions to DM from sources other than the feed itself, but true digestibility could not be calculated easily by this method.

### **6.2.6 Calculation of cumulative methane production**

Carbon dioxide and methane volumes were calculated from the total gas volumes and percentages of each gas. Cumulative total gas, carbon dioxide and methane

production values were calculated for each sample. These values were multiplied by the apparent DM digestibility values to give cumulative total gas, carbon dioxide and methane values per gram of apparently digested DM.

### 6.2.7 Data analysis

GenStat 16th edition (2013) was used to fit the France *et al.* (1993) model ( $y = A - BQ^tZ^{\sqrt{t}}$ , where  $Q = e^{-b}$ ,  $Z = e^{-c}$ , and  $B = e^{bT+c\sqrt{T}}$ ) to the data. In this model,  $y$  represents cumulative methane production (ml),  $t$  is the incubation time (h),  $A$  is the asymptote for the methane pool size (ml),  $T$  is the lag-time before the rate of methane production increases, and  $b$  ( $\text{h}^{-1}$ ) and  $c$  ( $\text{h}^{-0.5}$ ) are rate constants.

Estimates of the model parameters  $A$ ,  $Q$ ,  $T$  and  $Z$  were determined by fitting the the France *et al.* (1993) model to the cumulative methane production data. These estimated parameters were then used to determine fractional rates of degradation (Equation 1), total methane production in the system (asymptote), and cumulative methane production at any time point within the 120 hour measurement period (Equation 2).

#### Equation 1:

Fractional rate of degradation ( $\text{h}^{-1}$ ) =  $b + c/(2\sqrt{t})$

#### Equation 2:

Cumulative methane production at  $t$  hours (ml) =  $\text{Constant}(1-Q^t)$

GenStat 16th edition (2013) was used to perform repeated measures analysis of variance to compare samples in terms of total methane production potential in the system (ml/g apparently digested DM), cumulative methane production at 16, 24, 36

and 48 hours, and fractional rate of degradation. Functional bisector regression was performed using GenStat to correlate total methane production potential, cumulative methane production at 16, 24, 36 and 48 hours and fractional rate of degradation with analytical chemistry results, such as feed neutral detergent fibre (NDF) and water soluble carbohydrate (WSC) concentration values.

Further details regarding data analysis specific to each experiment are given in the relevant sections of this chapter.

## **6.3 *In vitro* gas production from upland plant species**

### **6.3.1 Introduction**

The aim of this study was to compare total gas production and methane production from fourteen different plant species collected on the same day in July 2011. The samples were selected in order to be representative of upland plants at a time of year during which they would be likely to be undergoing vegetative growth. This should ensure that the plants were relatively comparable in terms of their growth phase. The objectives were to measure the gas produced by each sample and the concentrations of methane and carbon dioxide in the gas produced, and to estimate DM digestibility for each sample in order to compare the gas and methane production per gram of dry matter digested between samples.

## **6.3.2 Materials and methods**

### **6.3.2.1 Samples used**

The samples used in this experiment were the upland plant samples collected from Pwllpeiran in July 2011 (numbers 43 to 56 in Table 14). A standard grass silage sample was also used (Section 6.1.4.2) as a control. Each sample was used in triplicate and blank samples were also analysed (Section 6.1.4).

### **6.3.2.2 Rumen inoculum**

The rumen fluid for this experiment was a mixture from three fistulated cows. The cows were fed a diet of grass silage and rumen contents was collected in the morning before feeding. The rumen contents were squeezed through a metal sieve to obtain the rumen fluid. This was then transferred into a pre-warmed Dewar flask.

### **6.3.2.3 Method**

The method used is described in detail in Section 6.2.

### **6.3.2.4 Calculations and model fitting**

Cumulative volumes of methane per gram of dry matter digested were calculated using the percentages of methane in gas produced, total volumes of gas measured at each time point and the estimates of digestibility for each sample. The results were also corrected for blanks. Results are also presented without correction for digestibility, showing total volumes of methane produced per g DM present in gas production bottles. The calculations are detailed in Section 6.2.6. The France *et al.* (1993) model was fitted to the data as explained in Section 6.2.7. Standard errors were also calculated.

#### **6.3.2.5 Statistical analysis**

The model parameters as fitted to each sample dataset were compared, these being total cumulative gas production, fractional rate of degradation and gas production at different time points (16, 24, 36 and 48 hours). These values were calculated from the modelled gas production values for these time points. The time points were chosen as being representative of possible rumen retention times, which can vary depending on feed offered; for example, poor quality forages are associated with greater rumen retention times than good quality forages (Sriskandarajah, 1981). Repeated measures analysis of variance (ANOVA) using species as a treatment, performed using GenStat 16th edition (2013), was used to compare the different upland plants, and different groups of upland plants, in terms of these parameters. These parameters were also correlated with NDF, ADF and WSC using functional bisector regression in GenStat.



### 6.3.3 Results

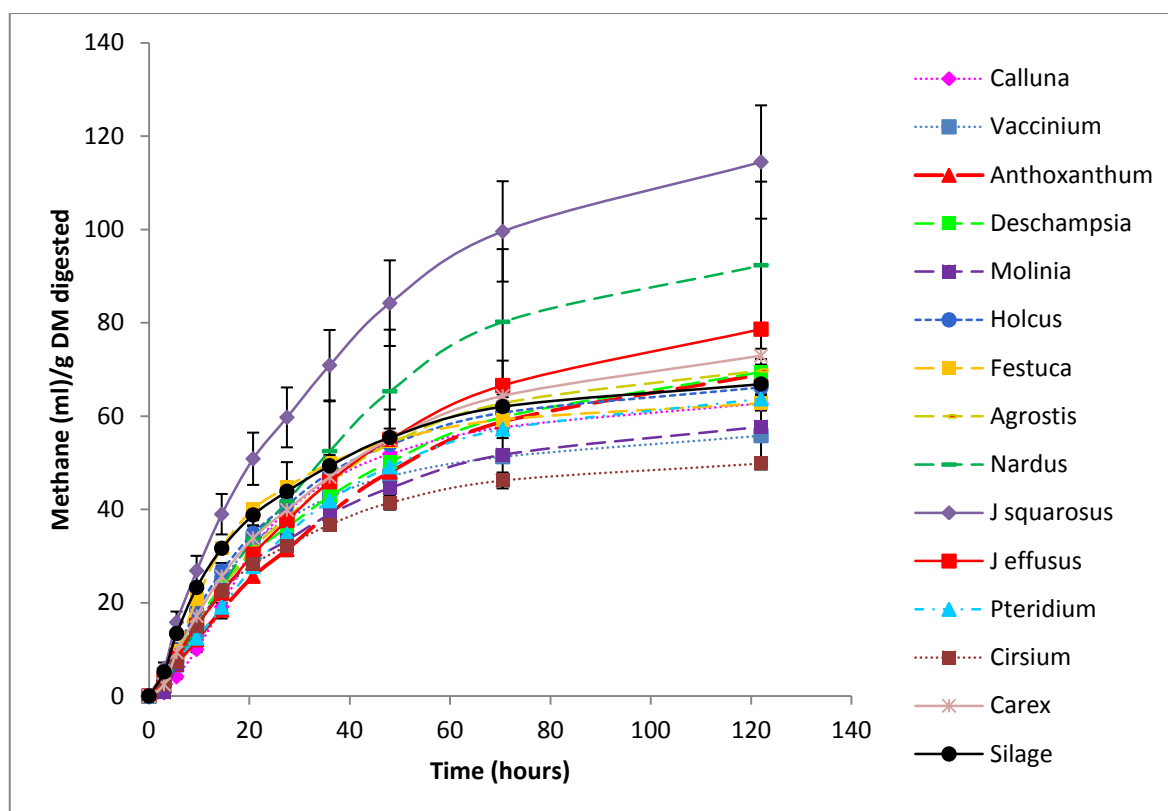
#### 6.3.3.1 Methane production curves

Figure 19 shows the methane production curves for all fourteen samples, with standard error bars, using data corrected for digestibility (ml methane per g apparently digested DM). This shows that there are some clear differences between some of the samples in terms of methane produced per gram of dry matter digested, with *Juncus squarosus* producing the largest volume of methane and *Cirsium palustre* producing the smallest volume of methane. Methane production curves for each sample, not corrected for digestibility (ml methane per g DM) are presented in Figure 20. Without correction for digestibility, *Festuca spp.* produced the largest volume of methane and *Calluna vulgaris* produced the smallest volume of methane.

Due to the large number of samples, and considerable overlap between samples, this is not a particularly clear method of displaying the data. Therefore, in Figure 21 and Figure 22 (using data corrected and not corrected for digestibility, respectively), the samples have been grouped according to type (see Table 14). This is a much clearer representation of the data. It clearly shows that the forbs (*Calluna vulgaris*, *Vaccinium myrtillus* and *Cirsium palustre*) produced the smallest volumes of methane and the rushes (*Juncus effusus* and *Juncus squarosus*) produced the most per gram of dry matter digested (Figure 21). When data were not corrected for digestibility, the forbs also produced the smallest volume. However, the standard silage sample and grasses produced the largest volumes of methane (Figure 22).

More detailed statistical analysis was used to determine which of the samples produced significantly more and less methane, in terms of total methane produced in

the system, methane produced at typical rumen retention times and fractional rates of degradation (Table 15 and Table 16).



**Figure 19: Methane production curves of fourteen upland plant species corrected for digestibility**

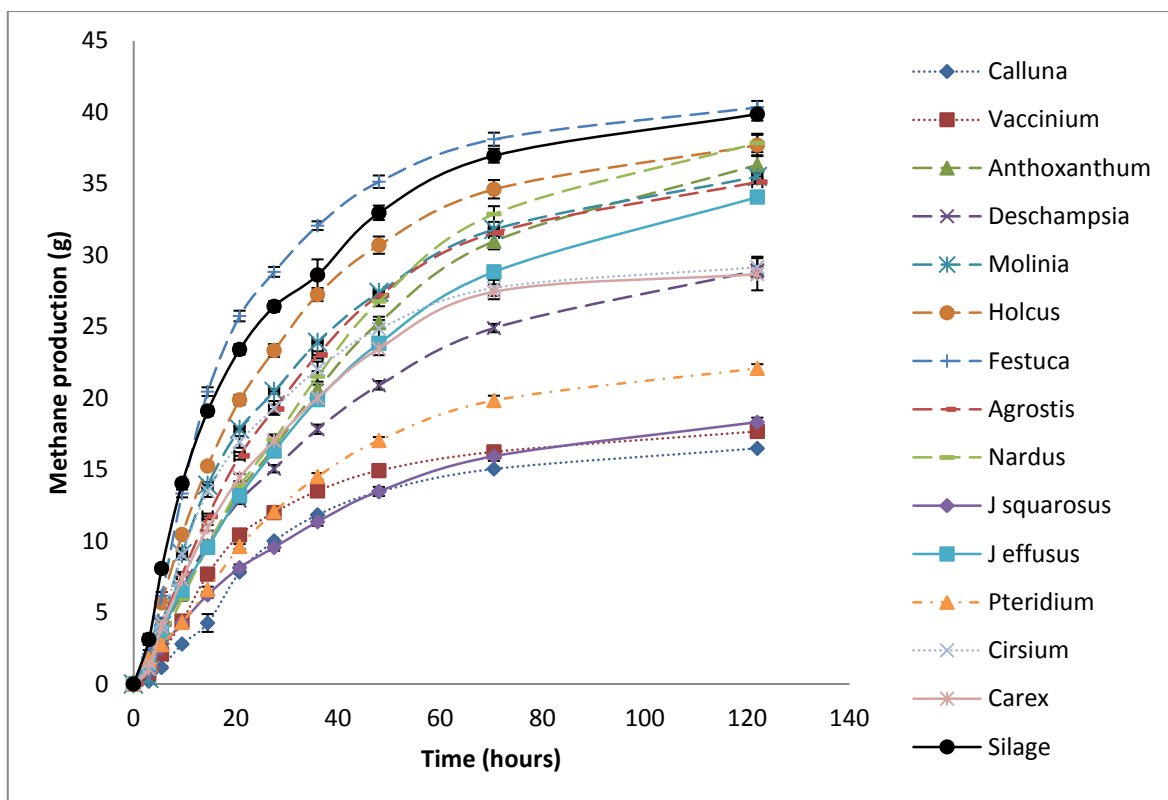
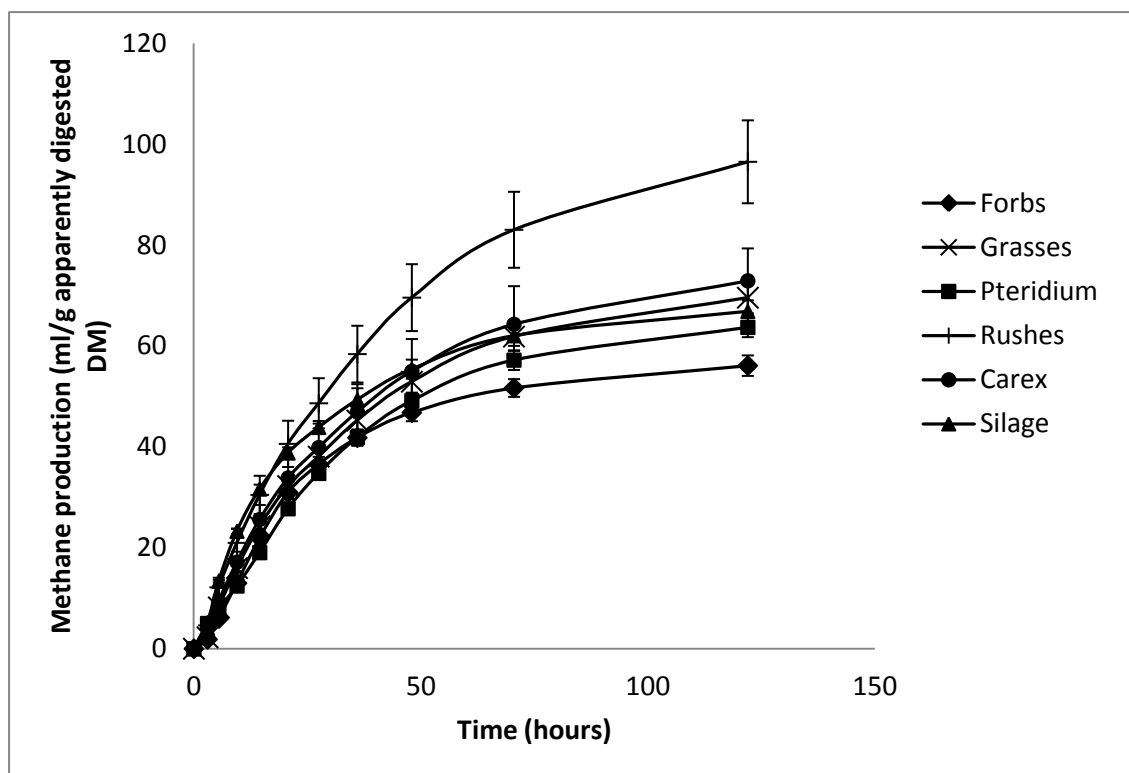
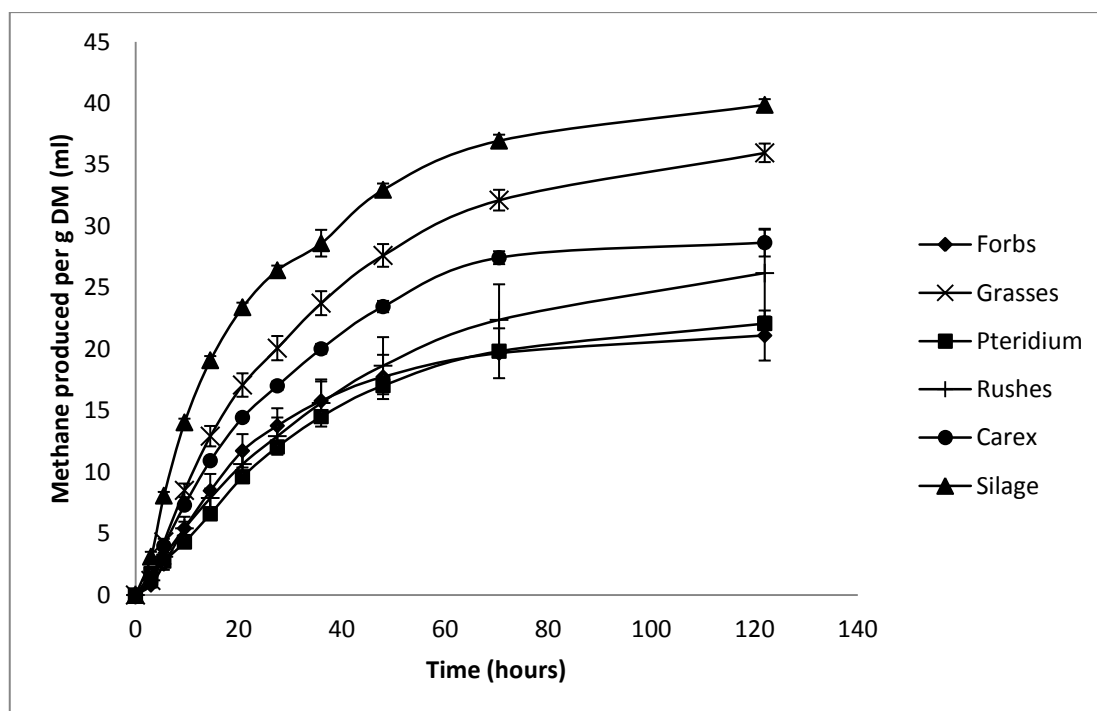


Figure 20: Methane production curves of fourteen upland plant species uncorrected for digestibility



**Figure 21: Methane production curves for groups of upland plant species corrected for digestibility**

Forbs include *Calluna vulgaris*, *Cirsium palustre*, and *Vaccinium myrtillus*. Grasses include *Agrostis spp.*, *Anthoxanthum odoratum*, *Deschampsia cespitosa*; *Festuca spp.*, *Molinia caerulea*; and *Nardus stricta*. Rushes include *Juncus effusus* and *Juncus squarosus*.



**Figure 22: Methane production curves for groups of upland plant species uncorrected for digestibility**

Forbs include *Calluna vulgaris*, *Cirsium palustre*, and *Vaccinium myrtillus*. Grasses include *Agrostis spp.*, *Anthoxanthum odoratum*, *Deschampsia cespitosa*; *Festuca spp.*, *Molinia caerulea*; and *Nardus stricta*. Rushes include *Juncus effusus* and *Juncus squarosus*.

### **6.3.3.2 Repeated measures ANOVA**

There were significant differences ( $P < 0.05$ ) between samples in terms of fractional rates of degradation, total methane production in the system, and methane production at 16, 24, 36 and 48 hours, when data were corrected for digestibility (Table 15) and when not corrected for digestibility (Table 16). These were taken as a range of possible rumen retention times, as rumen retention times are variable depending on animal and diet.

**Table 15: Table of mean methane production parameters, standard error of the difference and significance of differences between plant species in terms of methane production per gram of apparently digested dry matter**

Methane production parameter	Species means															SED	p
	<i>Agrostis</i>	<i>Anthoxanthum odoratum</i>	<i>Calluna vulgaris</i>	<i>Carex spp.</i>	<i>Cirsium palustre</i>	<i>Deschampsia cespitosa</i>	<i>Festuca spp.</i>	<i>Holcus lanatus</i>	<i>Juncus effusus</i>	<i>Juncus squarrosus</i>	<i>Molinia caerulea</i>	<i>Nardus stricta</i>	<i>Pteridium aquilinum</i>	<i>Vaccinium myrtillus</i>	Standard silage sample		
Total methane (ml/g DM digested)	67.5	70.4	53.7	65.9	46.3	69.4	58.4	63.2	77.9	105.3	55.0	99.1	61.1	51.1	63.1	9.15	<0.001
Fractional rate of degradation (g/h)	0.032	0.024	0.045	0.037	0.046	0.024	0.059	0.039	0.024	0.025	0.035	0.026	0.031	0.054	0.037	0.002	<0.001
Methane at 16 hours (ml/g DM digested)	28.0	22.8	29.3	30.3	24.7	22.2	36.7	29.8	25.3	34.7	24.1	35.2	24.5	30.7	27.7	3.85	0.008
Methane at 24 hours (ml/g DM digested)	37.3	31.2	37.3	39.7	31.6	30.4	45.2	38.9	34.6	47.5	31.9	47.8	32.8	38.2	36.6	5.00	0.013
Methane at 36 hours (ml/g DM digested)	47.3	41.1	44.6	49.4	38.0	40.2	52.1	48.1	45.6	62.5	40.0	62.2	41.8	44.6	45.9	6.21	0.009
Methane at 48 hours (ml/g DM digested)	54.0	48.6	48.7	55.4	41.6	47.5	55.4	53.8	53.8	73.6	45.2	72.5	48.0	47.8	51.9	7.03	0.003

Abbreviations: DM, dry matter; SED, standard error of the difference.

**Table 16: Table of mean methane production parameters, standard error of the difference and significance of differences between plant species without correction for digestibility**

Methane production parameter	Species means															SED	p
	<i>Agrostis</i>	<i>Anthoxanthum odoratum</i>	<i>Calluna vulgaris</i>	<i>Carex spp.</i>	<i>Cirsium palustre</i>	<i>Deschampsia cespitosa</i>	<i>Festuca spp.</i>	<i>Holcus lanatus</i>	<i>Juncus effusus</i>	<i>Juncus squarrosus</i>	<i>Molinia caerulea</i>	<i>Nardus stricta</i>	<i>Pteridium aquilinum</i>	<i>Vaccinium myrtillus</i>	<i>Standard silage sample</i>		
Total methane (ml/g DM)	35.8	38.9	16.4	29.4	28.9	29.9	39.1	37.6	36.2	19.1	35.4	40.2	22.8	17.2	39.6	0.75	<0.001
Fractional rate of degradation (g/h)	0.035	0.025	0.054	0.039	0.049	0.026	0.064	0.041	0.024	0.025	0.037	0.029	0.033	0.060	0.035	0.003	<0.001
Methane at 16 hours (ml/g DM)	14.9	12.5	9.0	13.4	15.4	10.2	24.6	17.7	11.5	6.3	15.5	14.2	9.1	10.2	17.3	0.56	<0.001
Methane at 24 hours (ml/g DM)	19.8	17.2	11.4	17.5	19.7	13.9	30.3	23.1	15.8	8.6	20.5	19.3	12.2	12.8	22.9	0.64	<0.001
Methane at 36 hours (ml/g DM)	25.1	22.7	13.7	21.8	23.7	18.2	34.9	28.6	20.9	11.4	25.7	25.2	15.6	14.9	28.7	0.67	<0.001
Methane at 48 hours (ml/g DM)	28.6	26.8	14.9	24.5	26.0	21.3	37.1	32.0	24.7	13.4	29.1	29.3	17.9	16.0	32.5	0.66	<0.001

Abbreviations: DM, dry matter; SED, standard error of the difference.

### 6.3.3.3 Functional bisector regression with % NDF, ADF and WSC

The percentages of NDF, ADF and WSC in each species sample analysed are shown in Table 17.

**Table 17: Percentages of NDF, ADF and WSC in each species sample**

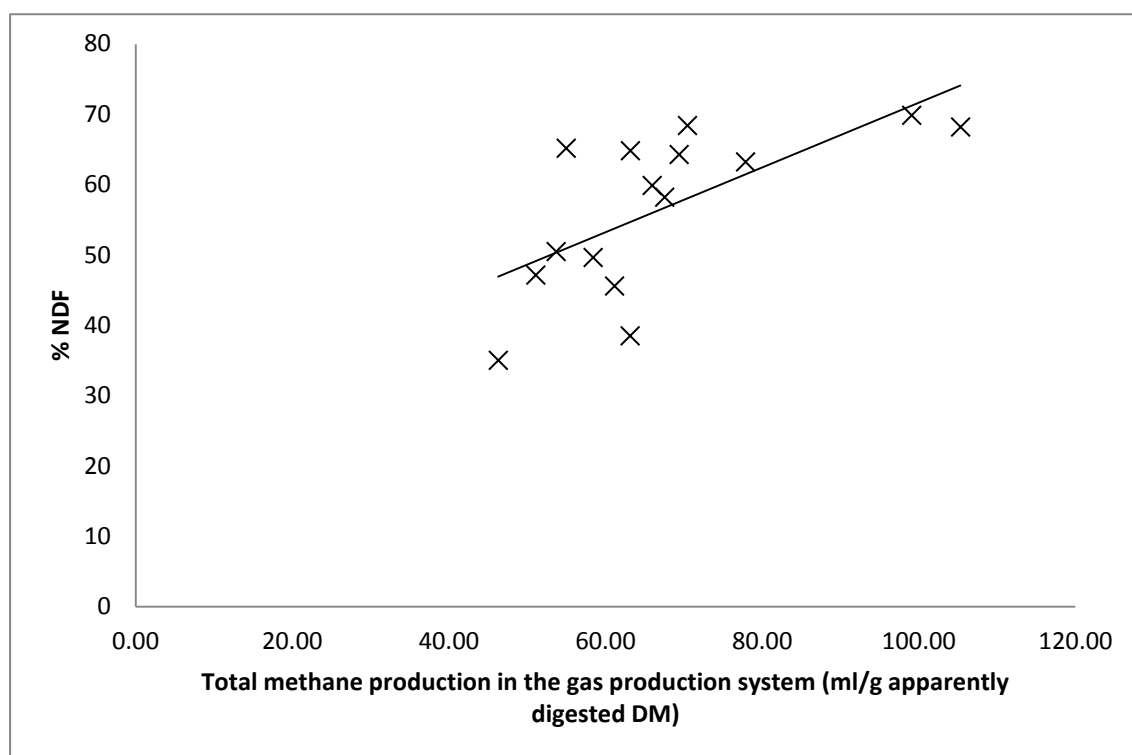
Species	% NDF	% ADF	% WSC
<i>Calluna vulgaris</i>	50.47	44.13	5.58
<i>Vaccinium myrtillus</i>	47.13	34.91	7.29
<i>Anthoxanthum odoratum</i>	68.39	36.11	8.49
<i>Deschampsia cespitosa</i>	64.27	36.02	7.86
<i>Molinia caerulea</i>	65.16	29.38	7.18
<i>Holcus lanatus</i>	64.81	28.00	11.96
<i>Festuca spp.</i>	49.63	26.02	21.32
<i>Agrostis spp.</i>	58.20	27.47	10.88
<i>Nardus stricta</i>	69.84	33.95	6.37
<i>Juncus squarrosus</i>	68.18	31.84	6.92
<i>Juncus effusus</i>	63.22	28.05	12.33
<i>Pteridium aquilinum</i>	45.57	30.71	9.81
<i>Cirsium palustre</i>	35.01	31.17	10.30
<i>Carex spp.</i>	59.87	25.69	10.71
Silage	38.49	20.78	22.73

Figure 23 shows a significant positive correlation

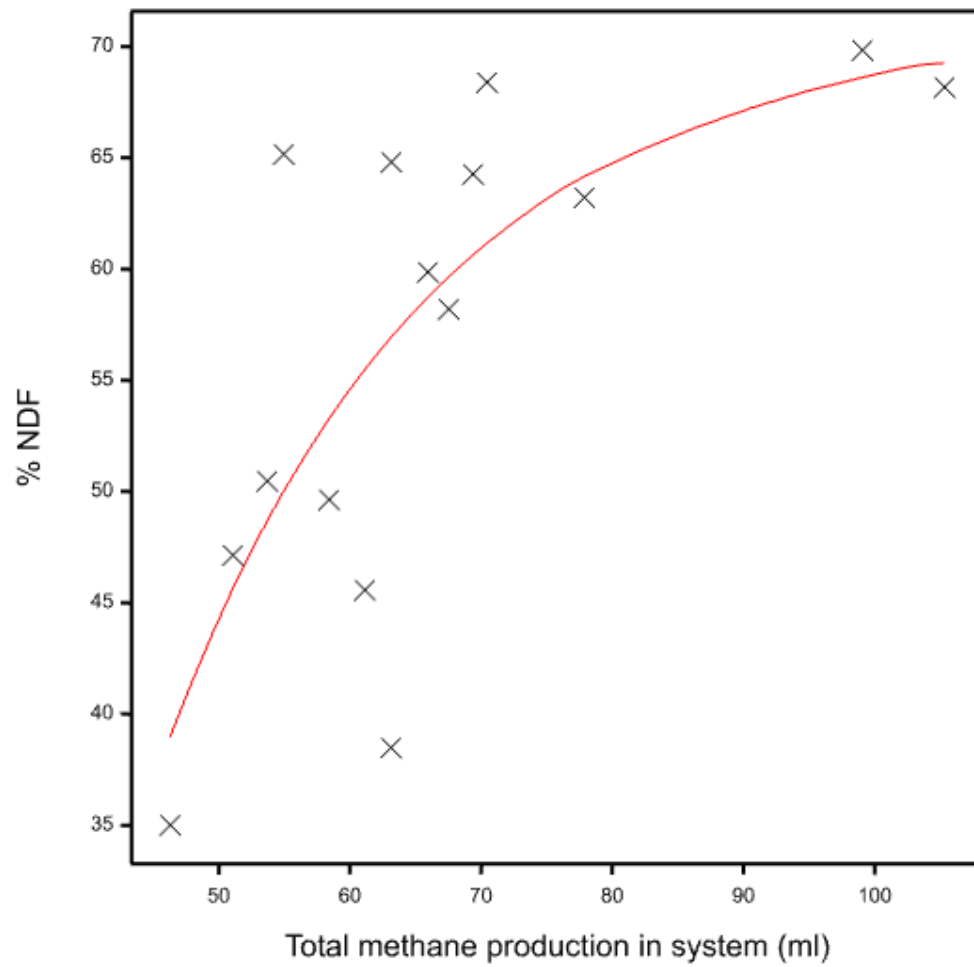
( $y=8.94(s.e.13.34)+0.7089(s.e.0.2155)x$ ) ( $R=0.67$ ;  $P<0.01$ ) between total methane production in the *in vitro* system (ml/g apparently digested DM) and percentage of NDF in the samples. Despite the linear relationship between total methane production and percentage of NDF in the sample, the plotted data points shown in Figure 23 appeared to be potentially better described by a curve than a straight line. Non-linear regression was therefore conducted in GenStat 16th edition (2013) to explore this relationship further: the resulting relationship ( $y=71.07(s.e.9.39)+ -305(s.e.458) X (0.9525(s.e.0.0330))x$ ;  $R=0.68$ ;  $P=0.01$ ) is shown in Figure 24. There were no significant correlations between percentage of ADF (Figure 25) or WSC (Figure 26) and total methane production in the system ( $P=0.994$  and  $P=0.452$ , respectively).



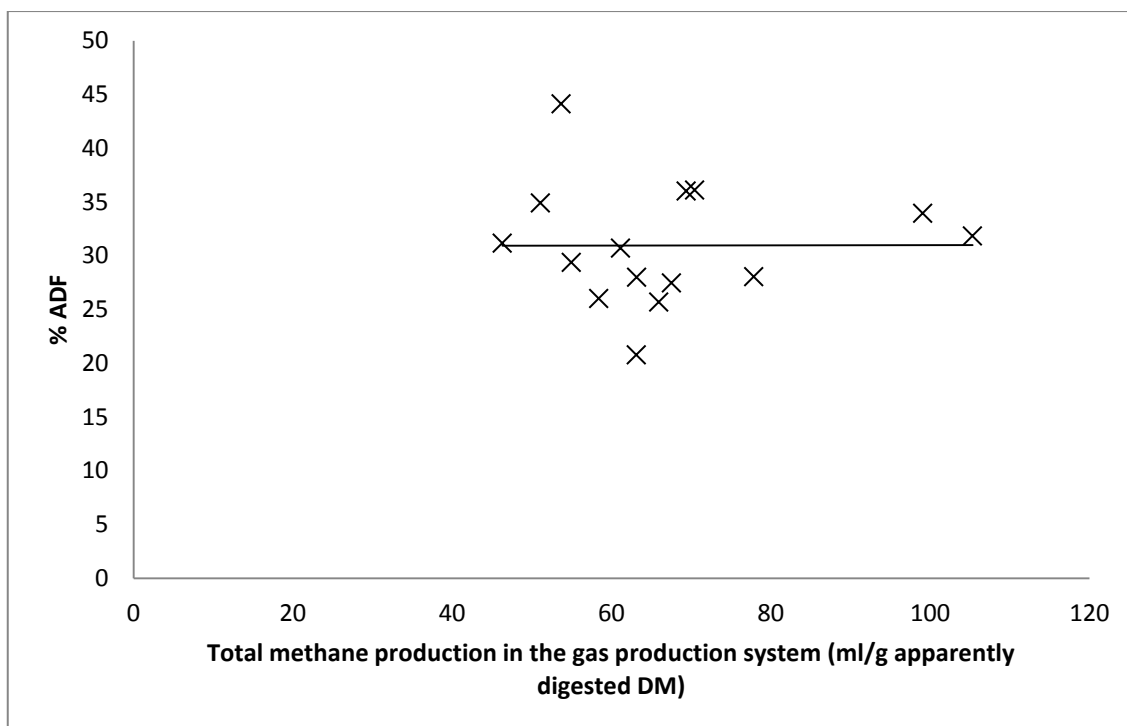
When methane production was not corrected for apparent DM digestibility, the relationships between total methane production (ml/g DM) and NDF, ADF, and WSC were not statistically significant.



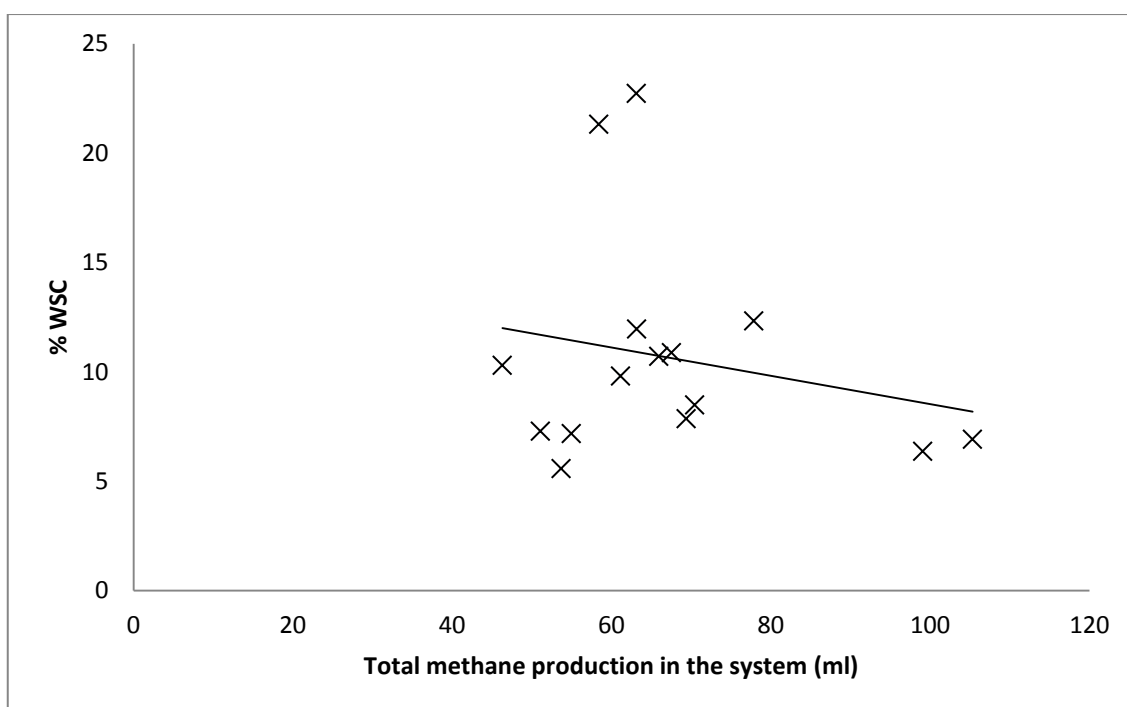
**Figure 23: Functional bisector relationship between total methane production (ml/g apparently digested DM) and percentage of neutral detergent fibre (NDF) in the sample**



**Figure 24: Non-linear relationship between total methane production in the gas production system (ml/g apparently digested DM) and the percentage of NDF in the sample**



**Figure 25: Functional bisector relationship between total methane production in the gas production system (ml/g apparently digested DM) and percentage of acid detergent fibre (ADF) in the sample**



**Figure 26: Functional bisector relationship between total methane production in the gas production system (ml/g apparently digested DM) and percentage of water soluble carbohydrate (WSC) in the sample**

### 6.3.4 Discussion

In accordance with previous studies (Theodorou *et al.*, 1994; Davies *et al.*, 2000), the results of the ANOVA demonstrate that the *in vitro* gas production technique can be used effectively to differentiate between upland plants in terms of total methane production in the *in vitro* system, fractional rate of degradation, and methane produced at certain time points, which would ideally be feed rumen retention times. The experiment presented here therefore adds to the existing evidence that *in vitro* gas production can be used as a means of estimating methane emissions by animals fed on particular diets. The use of the technique is explored further in the following sections of this chapter is validated in Chapter 7 against daily methane emissions data from sheep, as measured in methane chambers.

The grasses analysed in this section were a particularly divergent group, ranging from the relatively wiry and unpalatable *Nardus stricta* (Welch, 1986) to more nutritious and palatable grasses, such as *Festuca spp.* Without correction for digestibility, the grass producing the highest volume of methane was *Holcus lanatus* and the grass producing the lowest volume of methane was *Deschampsia cespitosa*. However, when corrected for digestibility, the grass producing the highest volume of methane was *Nardus stricta*, and the grass producing the lowest volume was *Molinia caerulea*. This suggests that some of the differences in methane production between the grasses is due to digestibility. However, some grasses, such as *Nardus stricta*, produced relatively high volumes of methane when the data were corrected or uncorrected for digestibility. This suggests that additional composition factors may also contribute to the relative methane production potentials of the grasses.

There was a significant linear and a significant non-linear relationship between the total methane production in the system (ml/g apparently digested DM) and the NDF concentration in samples. This was expected as previous studies have shown significant linear relationships between the mass of NDF digested and gas produced by samples *in vitro* (Calabro *et al.*, 2002; Doane *et al.*, 1997), although in the experiments presented in this thesis the NDF was not digested NDF but NDF concentration of plant samples. However, when methane production was not corrected for DM digestibility, no significant relationship was found between *in vitro* methane production (ml/g DM) and the percentage of NDF in the sample. No relationship was found between the percentage of ADF in the sample and total methane production in the system (either ml/g apparently digested DM or ml/g DM). This may be due to this portion of the plant material remaining largely undigested (Van Soest, 1994) and therefore having little impact on methane production potential in the *in vitro* system.

Similarly, no significant relationship was shown between percentage of WSC and total methane production in the system. This is to be expected: higher concentrations of more readily digestible carbohydrates have been shown to reduce methane production compared with more fibrous feeds (Benchaaar *et al.*, 2001).

Increased concentration of NDF in a sample may increase the rumen retention time of a feed (Sriskandarajah, 1981), providing more time for ruminal fermentation: plant samples that are not readily fermentable may produce as much methane as less fibrous plant samples when rumen retention time is considered. This is not taken into account in the *in vitro* gas production results, although, if a typical rumen retention time for a particular feed were known, it would be possible to calculate the methane

produced *in vitro* by that feed in that particular time. Menke *et al.* (1979) found that 24 hours was not a long enough incubation time to allow for the fermentation of more fibrous feeds. The time points that were modelled in the analyses (16, 24, 36 and 48 hours) reflect the diversity of plant samples used and potential rumen retention times for each sample.

### **6.3.5 Conclusion**

In conclusion, the experiment presented here shows that upland plants collected at the same time of year were distinguishable based on their gas production profiles. The group of plants associated with the highest methane production was the rushes, including *Juncus effusus* and *Juncus squarosus*, with the forbs (*Calluna vulgaris* and *Vaccinium myrtillis*) associated with the lowest methane production. Neutral detergent fibre concentration of plant samples was shown to be a reasonable predictor for *in vitro* methane production from plant samples, which is consistent with previous studies showing that more fibrous feeds are associated with increased methane production (Benchaar *et al.*, 2001).

## **6.4 *In vitro* gas production by upland plants collected at different times of year**

### **6.4.1 Introduction**

The aim of this study was to investigate the effect of time of sample collection on methane production from plant samples. Sheep often graze upland pastures for several months of the year and the composition of grazing material changes throughout the year. This may affect the methane production potentials of the grazed material. In this study, *in vitro* gas production was used to compare methane production potentials of samples of *Molinia caerulea* and *Calluna vulgaris*, collected at different times of year. In some cases, plant material was senescent at certain times of year. Senescent material would be less palatable and less nutritious for grazing animals, although this material would still be eaten by grazing animals. It was therefore still relevant to obtain methane production potentials for plant samples that are partially, or entirely, senescent.

## **6.4.2 Materials and methods**

### **6.4.2.1 Samples used**

The samples used in this study were *Molinia caerulea* and *Calluna vulgaris*, collected in April, June, August, October, January and March. These were sample numbers 1, 5, 29, 33, 57, 61, 85, 89, 113, 117, 141 and 145 in Table 14. A standard grass silage sample was also used (Section 6.1.4.2). Each sample was analysed in triplicate with three blanks, as described in Section 6.1.4. Approximately one gram of freeze-dried substrate was used in each gas production bottle, though the exact weights were recorded.

### **6.4.2.2 Rumen inoculum**

The rumen inoculum for this study was taken from the same three fistulated cows as used in Section 6.3. The cows were fed on a diet of grass silage and rumen fluid was collected in the morning before feeding.

### **6.4.2.3 Method**

The method used is described in detail in Section 6.2.

### **6.4.2.4 Calculations and model fitting**

Cumulative volumes of methane per gram of dry matter digested were calculated using the concentration of methane in gas produced, total volumes of gas at each time point and the estimates of digestibility for each sample. The results were also corrected for blanks. The calculations are detailed in Section 6.2.6. The France *et al.* (1993) model was fitted to the data as explained in Section 6.2.7. Standard errors were also calculated.



#### **6.4.2.5 Statistical analysis**

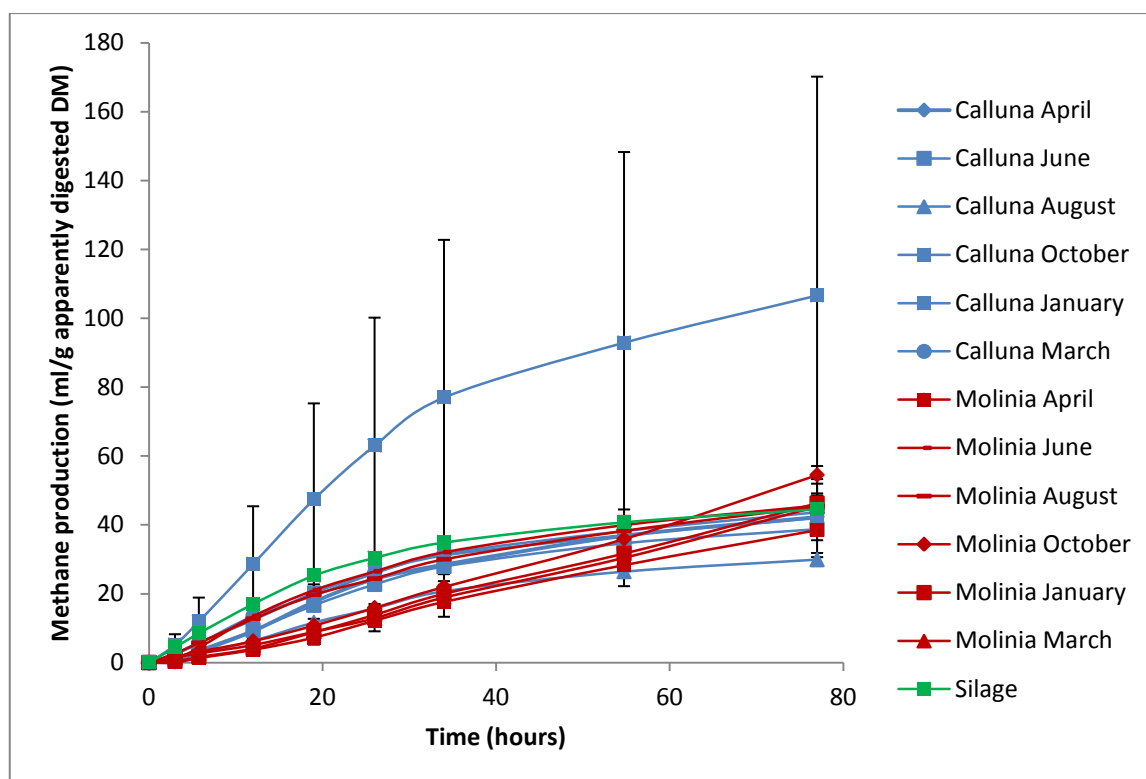
The samples were then compared in terms of the parameters of the model, which were total cumulative gas production, fractional rate of degradation and gas production at different time points (16, 24, 36 and 48 hours), which are possible rumen retention times. Analysis of variance (ANOVA), using species as a treatment structure, performed using GenStat 16th edition (2013), was used to compare the different upland plants, and different groups of upland plants, in terms of these parameters. Unbalanced ANOVA was used to compare the samples; some of the samples were excluded from the analysis as the France *et al.* (1993) model could not be fitted to their data due to the plant material be senescent. These parameters were also correlated with NDF, ADF and WSC using functional bisector regression in GenStat 16th edition (2013).

### 6.4.3 Results

#### 6.4.3.1 Methane production curves

Figure 27 shows methane production curves corrected for dry matter apparently digested and blanks. The France *et al.* (1993) model did not fit particularly well to some of the data, for example, the *Molinia caerulea* samples not collected during the summer months. The methane production curves are therefore not the expected shape. This is probably because the plant material collected during the colder months was largely senescent, affecting the digestibility of the samples. The gas production curves for *Molinia caerulea* collected in April, October, January and March do not appear to reach a peak within the experimental period. However, the experimental period is longer than any expected rumen retention time. These samples, therefore, appear to have been particularly indigestible. This was not the case for *Calluna vulgaris*, which is evergreen; samples were therefore not made up of senescent plant material. The *Calluna vulgaris* collected in January produced a large amount of methane per gram of digested DM. The standard errors for this sample were also unusually large. Returning to the raw data, one of the replicates for this sample appeared to be particularly indigestible and was apparently anomalous (>2 standard deviations from the mean of the group in terms of cumulative gas production) with the other two replicates. This replicate was therefore removed from the data set, the result of which is shown in Figure 28. Without this replicate, the *Calluna vulgaris* sample from January had a gas production curve much closer in character to those of the other *Calluna vulgaris* samples. Month of collection did appear to have a considerable impact on the methane production potential of the plants. These

differences do not seem to be consistent between species and may be related to the digestibility of particular species at particular times of year.



**Figure 27: Methane production curves of different species at different times of year (including anomaly)**

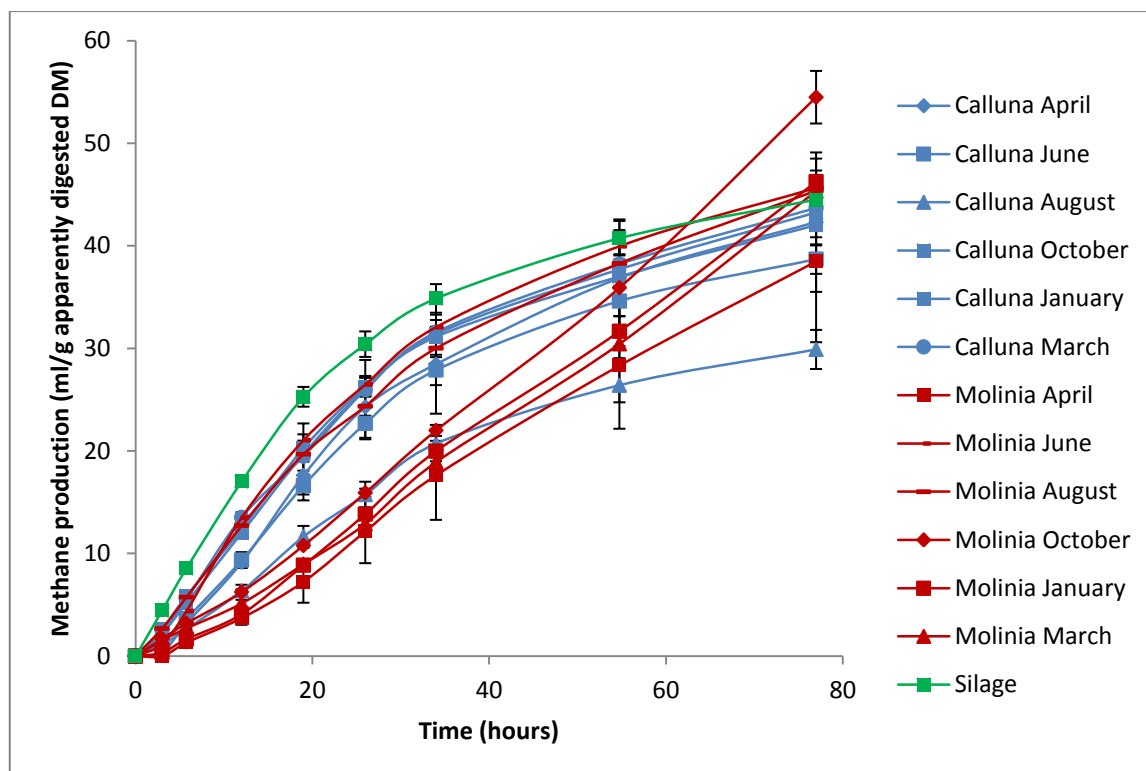


Figure 28: Methane production curves of different species at different times of year (excluding anomaly)

#### 6.4.3.2 Unbalanced ANOVA

The France *et al.* (1993) model did not fit to several of the gas production curves for the *Molinia caerulea* samples, which were therefore not included in the ANOVA: mean values for modelled total methane production in the system, fractional rate of degradation, and methane production at 16, 24, 36 and 48 hours are presented in Table 18. In terms of fractional rate of degradation, there were significant differences between species ( $P=0.006$ ) and months of sample collection ( $P=0.027$ ), but no significant interaction between them ( $P=0.233$ ). There were no significant differences between species or between months in terms of total methane production in the system ( $P=0.940$  and  $P=0.207$ , respectively), or methane production at 16 ( $P=0.882$  and  $P=0.195$ , respectively), 24 ( $P=0.896$  and  $P=0.197$ , respectively), 36 ( $P=0.911$  and  $P=0.199$ , respectively) or 48 ( $P=0.922$  and  $P=0.200$ , respectively) hours. Similarly, there

were no significant interactions between species and months of sample collection in terms of total methane production in the system ( $P=0.798$ ), or methane production at 16 ( $P=0.967$ ), 24 ( $P=0.949$ ), 36 ( $P=0.924$ ) and 48 ( $P=0.901$ ) hours.

**Table 18: Modelled methane production parameters for samples taken at different times of year**

Methane production parameter	Month						Standard silage sample
	January	March	April	June	August	October	
Total methane (ml/g DM apparently digested)							
<i>Molinia caerulea</i>	*	*	*	55.9	60.2	*	53.1
<i>Calluna vulgaris</i>	121.2	55.3	44.5	49.2	41.4	51.7	
Fractional rate of degradation (g/h)							
<i>Molinia caerulea</i>	*	*	*	0.037	0.026	*	0.041
<i>Calluna vulgaris</i>	0.037	0.034	0.037	0.038	0.032	0.038	
Methane at 16 hours (ml/g DM apparently digested)							
<i>Molinia caerulea</i>	*	*	*	26.1	21.3	*	25.3
<i>Calluna vulgaris</i>	56.9	23.8	21.0	23.5	17.7	24.3	
Methane at 24 hours (ml/g DM apparently digested)							
<i>Molinia caerulea</i>	*	*	*	34.2	28.9	*	34.0
<i>Calluna vulgaris</i>	74.3	31.3	27.4	30.6	23.4	31.8	
Methane at 36 hours (ml/g DM apparently digested)							
<i>Molinia caerulea</i>	*	*	*	42.3	37.6	*	41.7
<i>Calluna vulgaris</i>	92.0	39.2	33.9	37.7	29.5	39.3	
Methane at 48 hours (ml/g DM digested)							
<i>Molinia caerulea</i>	*	*	*	47.4	43.9	*	46.2
<i>Calluna vulgaris</i>	103.1	44.4	37.9	42.1	33.6	44.0	

\* France *et al.* (1993) model does not fit to this data and these parameters can therefore not be modelled.

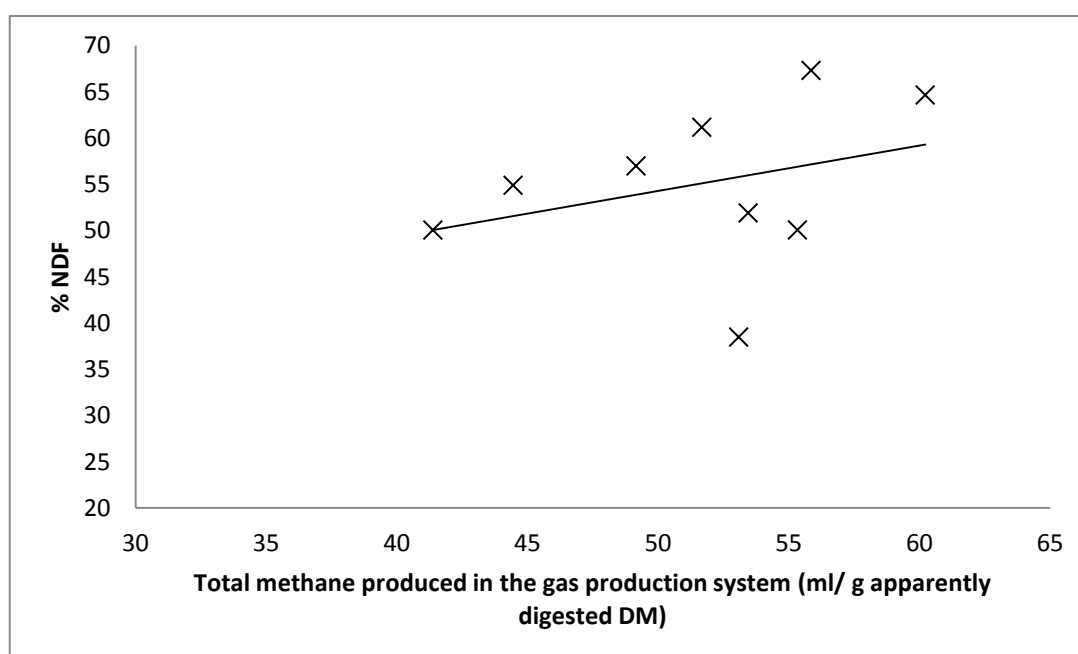
### 6.4.3.3 Functional bisector regression with % NDF, ADF and WSC

The percentages of NDF, ADF and WSC in each of the sample for which the model could be fitted are shown in Table 19.

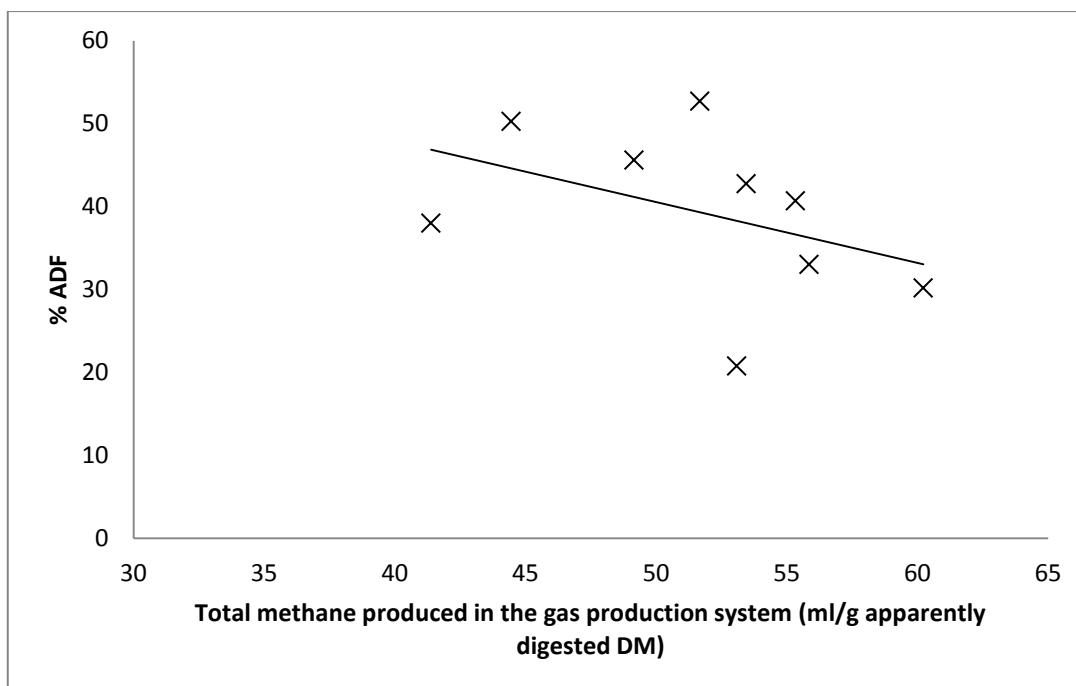
**Table 19: Percentages of NDF, ADF and WSC in samples**

Species	% NDF	% ADF	% WSC
<i>Calluna vulgaris</i> - January	51.91	42.76	8.59
<i>Calluna vulgaris</i> - March	50.07	40.7	7.55
<i>Calluna vulgaris</i> - April	54.90	50.28	4.14
<i>Calluna vulgaris</i> - June	56.99	45.61	4.15
<i>Calluna vulgaris</i> - August	50.06	38.01	6.52
<i>Calluna vulgaris</i> - October	61.17	52.71	4.86
<i>Molinia caerulea</i> - June	67.32	33.03	4.84
<i>Molinia caerulea</i> - August	64.66	30.19	8.47
Silage	38.49	20.78	22.73

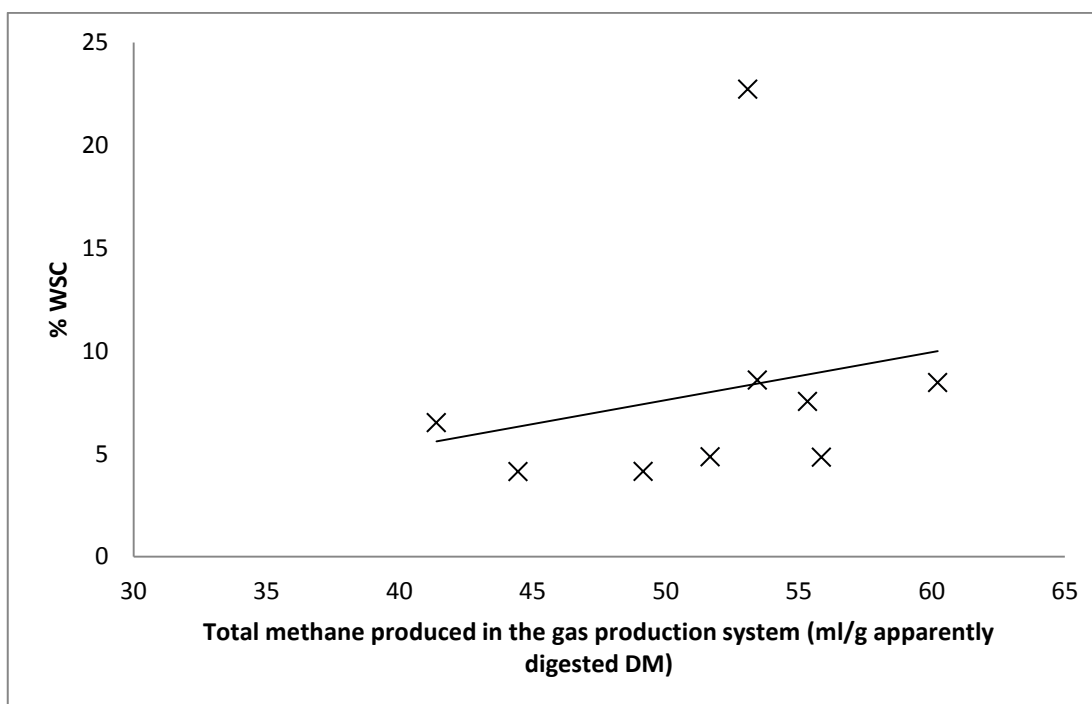
There were no significant correlations between total methane and percentages of NDF (Figure 29,  $P=0.393$ ), ADF (Figure 30,  $P=0.256$ ), or WSC (Figure 31,  $P=0.543$ ). As the only samples used were those with gas production data to which the France *et al.* (1993) model could be fitted, the analysis was limited.



**Figure 29: Functional bisector relationship between total methane production in the gas production system (ml/g apparently digested DM) and percentage of NDF in the sample**



**Figure 30: Functional bisector relationship between total methane production in the gas production system (ml/g apparently digested DM) and percentage of ADF in the sample**



**Figure 31: Functional bisector relationship between total methane production in the gas production system (ml/g apparently digested DM) and percentage of ADF in the sample**

#### 6.4.4 Discussion

The France *et al.* (1993) model did not fit to the gas production data from *Molinia caerulea* samples collected in April, October, January and March; *Molinia caerulea* was largely senescent for much of the year, which may explain the unusual gas production results. Although animals eat senescent plant material, *Molinia caerulea* has a low rate of leaf turnover, particularly when not grazed (Grant *et al.*, 1996). This means that the plant material becomes rank and less digestible, which may explain why the France *et al.* (1993) model did not fit well to the data when the plant material was senescent. *Molinia caerulea* is also a prolific upland plant and, as it is not useable as a grazing material when senescent, strategies such as burning and herbicide control have been investigated in order to control it (Ross *et al.*, 2004; Marrs *et al.*, 2004).

In relation to *Molinia caerulea*, the findings of this experiment are consistent with those of a previous experiment, in which good agreement was found between *in vitro* digestibilities of plant material collected in May and July, but not collected during September, when plant material would have been likely to be senescent (Grant and Campbell, 2006).

The France *et al.* (1993) model fitted to all gas production curves produced by *Calluna vulgaris* samples all produced gas production curves, although there was variation in terms of methane production parameters at different times of year. For example, the total methane produced in the system by *Calluna vulgaris* in January was more than double that produced in other months. Although *Calluna vulgaris* is an evergreen shrub, there were changes in chemical composition and fibre content over the course of the year. This could include changes in tannin concentration: seasonal changes in



tannin concentration in *Calluna vulgaris* have previously been shown to occur, with highest concentrations observed during the growing season (Gonzalez-Hernandez, 2003). The presence of condensed tannins has been associated with a reduction in *in vitro* methane production (Animut *et al.*, 2008; Frutos *et al.*, 2002; Tavendale *et al.*, 2005): it is therefore possible that variation in tannin concentration was partially responsible for seasonal changes in methane production potential of *Calluna vulgaris*.

#### **6.4.5 Conclusion**

There was considerable variation in *in vitro* gas production parameters from *Molinia caerulea* and *Calluna vulgaris* samples taken at different times of year. In the case of *Molinia caerulea*, this may have been due to plant material becoming rank and indigestible at certain times of year. Variation in tannin concentration could have had an impact on gas production parameters of *Calluna vulgaris* and the effect of condensed tannins on methane production potential is further explored in Section 6.5.

## **6.5 Comparison of *in vitro* methane production by mixtures of *Festuca spp.* and *Calluna vulgaris* in varying proportions**

### **6.5.1 Introduction**

The aim of this study was to compare *in vitro* methane production from two contrasting plant samples and to investigate the effect of mixing the two plant substrates in varying proportions. *Festuca spp.* was shown to have a higher fractional rate of degradation than *Calluna vulgaris* in the study described in Section two. *Calluna vulgaris* is known to have a high content of condensed tannins (6–12% DM) throughout the year (Frutos *et al.*, 2002), whereas *Festuca spp.* and other would be expected to have a condensed tannin content in the region of 0.12–0.47% DM (Montossi *et al.*, 1997). Condensed tannin concentrations in the samples used in this experiment were not evaluated due to time constraints.

Condensed tannins have been shown to effect organic matter degradation, particularly in relation to proteins, and gas production both *in vitro* (Animut *et al.*, 2008; Frutos *et al.*, 2002; Tavendale *et al.*, 2005) and *in vivo* (Animut *et al.*, 2008; Grainger *et al.*, 2009; Puchala *et al.*, 2005). This study was aimed at establishing whether there was any evidence that condensed tannins were responsible for any reduction in methane production, when the *Calluna vulgaris* containing these tannins was added to the *Festuca spp.* samples.

## **6.5.2 Materials and methods**

### **6.5.2.1 Samples used**

The samples used in this study were numbers 43 and 49 in Table 14. These were a sample of *Calluna vulgaris* and another of *Festuca spp.* collected in July 2011. Pure samples and mixtures of the two samples, in 20% increments were used. The samples were, therefore, as follows: 100% *Festuca*, 0% *Calluna vulgaris*; 80% *Festuca*, 20% *Calluna vulgaris*; 60% *Festuca*, 40% *Calluna vulgaris*; 40% *Festuca*, 60% *Calluna vulgaris*; 20% *Festuca*, 80% *Calluna vulgaris*; 0% *Festuca*, 100% *Calluna vulgaris*. The grass silage sample (Section 6.1.4.2) was also used as a standard and a blank, containing no substrate, was used. All samples were analysed in triplicate.

### **6.5.2.2 Method**

The method used is described in detail in Section 6.2. As in other experiments, the total substrate was about 1g, so the mixtures of substrates always added up to 1g.

### **6.5.2.3 Calculations and model fitting**

Cumulative volumes of methane per gram of dry matter digested were calculated using the percentages of methane in gas produced, total volumes of gas at each time point and the estimates of digestibility for each sample. The results were also corrected for blanks. The calculations are detailed in Section 6.2.6. The France *et al.* (1993) model was fitted to the data as explained in Section 6.2.7. Standard errors were also calculated. Cumulative volumes of methane per gram of dry matter, not corrected for digestibility, were also calculated and the France *et al.* (1993) model was also fitted to these values.

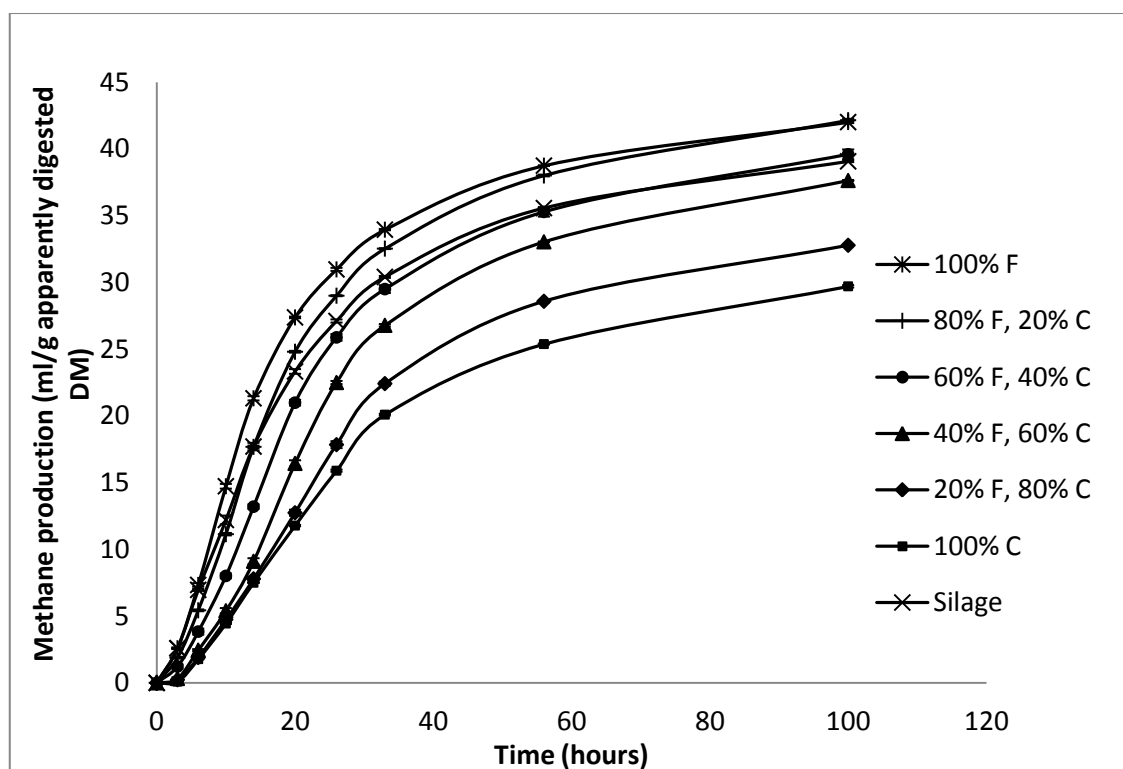
#### **6.5.2.4 Statistical analysis**

ANOVA with polynomial contrasts in GenStat 16th edition (2013) was used to compare the mixtures of samples in terms of fractional rate of degradation, total methane production in the system, and cumulative methane production at 16, 24, 36 and 48 hours, which are possible rumen retention times.

## 6.5.3 Results

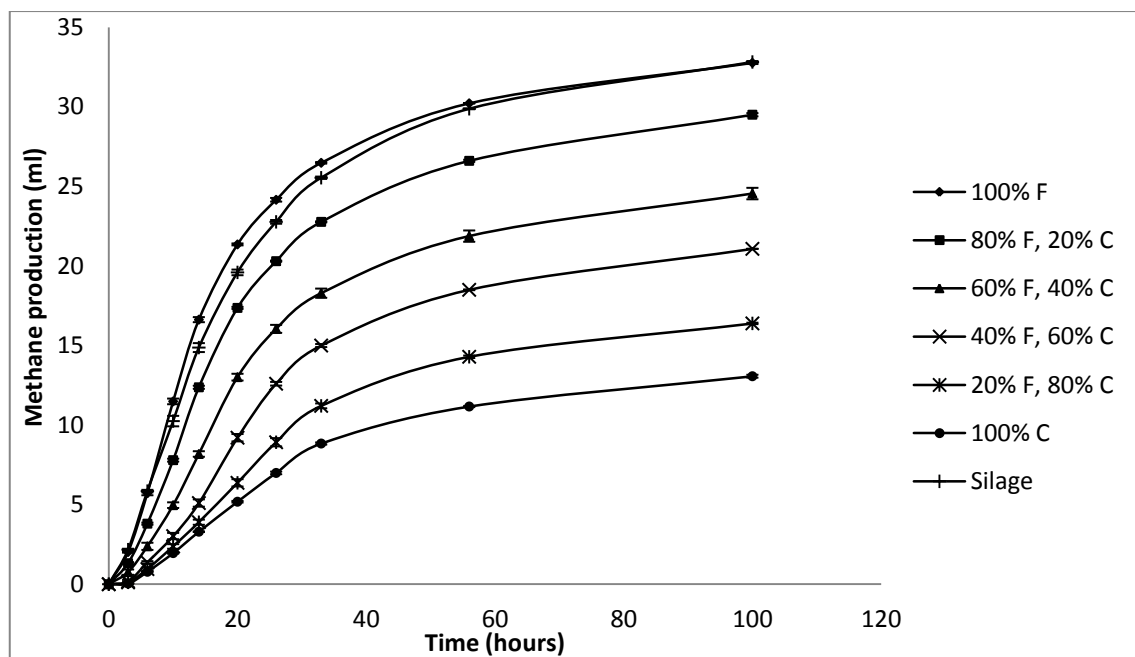
### 6.5.3.1 Methane production curves

Figure 32 shows the methane production curves for the various mixtures of samples corrected for digestibility. Methane production increased when the proportion of *Festuca spp.* in the mixture increased. Standard error bars are present in the figure, but they are too small to be clearly visible. Methane production curves for the mixtures of samples without correction for digestibility are presented in Figure 33. Without correction for digestibility, results were similar to those when corrected for digestibility in that increasing the proportion of *Festuca spp.* in the sample resulted in increased methane production. However, when not corrected for digestibility, differences in methane production between sample mixtures were greater than when corrected for digestibility.



**Figure 32: Methane production curves for varying proportions of *Calluna vulgaris* and *Festuca spp.***

Abbreviations: C, *Calluna vulgaris*; F, *Festuca spp.*



**Figure 33: Methane production curves for varying proportions of *Calluna vulgaris* and *Festuca spp.* without correction for digestibility**

Abbreviations: C, *Calluna vulgaris*; F, *Festuca spp.*

### 6.5.3.2 ANOVA with polynomial contrasts

Table 20 shows the mean total methane production, fractional rate of degradation, and cumulative methane production at 16, 24, 36 and 48 hours for each sample, using data corrected for digestibility. There were significant linear and quadratic effects of increasing the proportion of *Festuca spp.* in the samples for all of these parameters (Table 20). The means of the samples were plotted with a polynomial trendline, showing the quadratic effect of increasing the proportion of *Festuca spp.* An example is shown in Figure 34: this is the quadratic relationship between the proportion of *Festuca spp.* in the mixture and methane production at 36 hours. The results show that the increase in methane production was not directly proportional to the increase in proportion of *Festuca spp.*, but that even a slight increase in *Festuca spp.* causes a significant increase in methane production. Using data uncorrected for digestibility, there were significant linear effects of increasing the proportion of *Festuca spp.* in the

samples for all parameters (Table 21). However, there were not significant quadratic effects of increasing the proportion of *Festuca spp.* for total methane production, and methane production at 36 and 48 hours (Table 21). The linear relationship between the proportion of *Festuca spp.* in the mixture and methane production (uncorrected for digestibility) at 36 hours is presented in Figure 35.

**Table 20: Table of means and significance of linear and quadratic effects of increasing the proportion of *Festuca spp.* using data corrected for digestibility**

Methane production parameter	Percentage of <i>Festuca spp.</i> (%)						SED	P (linear)	P (quadratic)
	0	20	40	60	80	100			
Total methane (ml/g apparently digested DM)	30.5	33.8	38.2	39.5	41.6	41.1	0.51	<0.001	<0.001
Fractional rate of degradation (g/h)	0.041	0.042	0.048	0.054	0.059	0.068	0.0012	<0.001	<0.001
Methane at 16 hours (ml/g apparently digested DM)	15.9	18.0	22.1	24.1	26.5	28.0	0.33	<0.001	0.001
Methane at 24 hours (ml/g apparently digested DM)	20.4	23.0	27.7	29.9	32.5	33.7	0.37	<0.001	<0.001
Methane at 36 hours (ml/g apparently digested DM)	24.6	27.7	32.7	34.8	37.3	38.0	0.40	<0.001	<0.001
Methane at 48 hours (ml/g apparently digested DM)	27.1	30.3	35.3	37.2	39.6	39.7	0.42	<0.001	<0.001

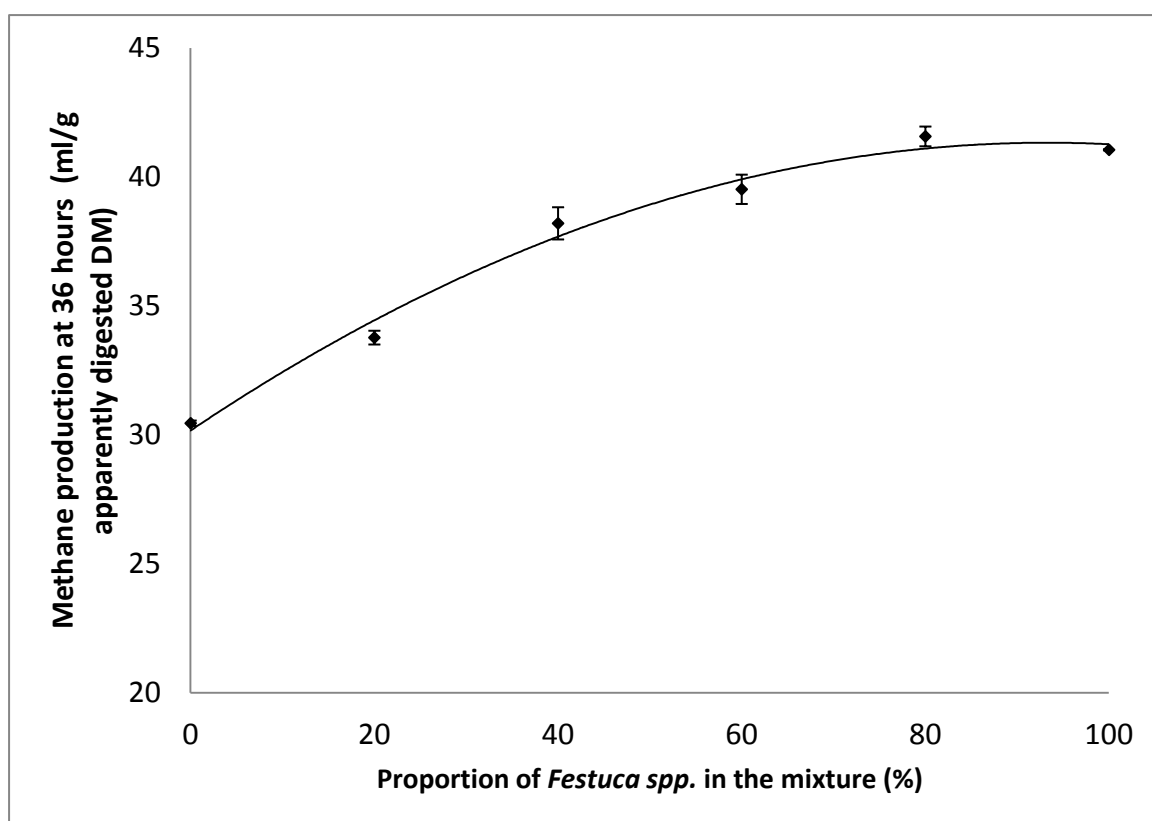
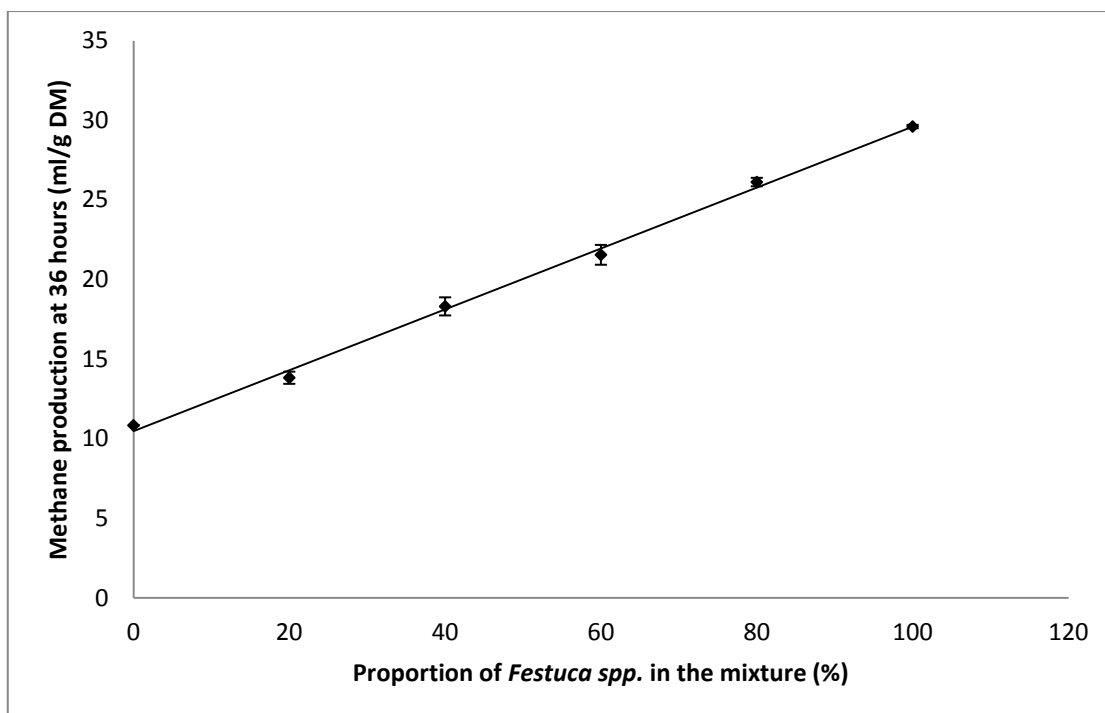


Figure 34: Quadratic relationship between proportion of *Festuca* spp. in the mixture and methane production at 36 hours, using data corrected for digestibility

Table 21: Table of means and significance of linear and quadratic effects of increasing the proportion of *Festuca* spp. using data uncorrected for digestibility

Methane production parameter	Percentage of <i>Festuca</i> spp. (%)						SED	P (linear)	P (quadratic)
	0	20	40	60	80	100			
Total methane (ml/g DM)	13.4	16.9	21.4	24.5	29.1	32.0	0.30	<0.001	0.23
Fractional rate of degradation (g/h)	0.018	0.021	0.027	0.033	0.041	0.053	0.001	<0.001	<0.001
Methane at 16 hours (ml/g apparently digested DM)	7.0	9.0	12.4	14.9	18.5	21.9	0.19	<0.001	<0.001
Methane at 24 hours (ml/g apparently digested DM)	9.0	11.5	15.5	18.5	22.7	26.3	0.22	<0.001	0.002
Methane at 36 hours (ml/g apparently digested DM)	10.8	13.8	18.3	21.6	26.1	29.6	0.24	<0.001	0.099
Methane at 48 hours (ml/g apparently digested DM)	11.9	15.2	19.8	23.0	27.7	31.0	0.26	<0.001	0.79





**Figure 35: Linear relationship between proportion of *Festuca spp.* in the mixture and methane production at 36 hours, using data uncorrected for digestibility**

#### 6.5.4 Discussion

There were significant linear and quadratic relationships between the proportions of the different plant material in the substrate mixtures and the cumulative methane production at 16, 24, 36 and 48 hours, total methane production in the system, and fractional rate of degradation. The significant quadratic relationships show that increasing the proportion of *Festuca spp.*, the substrate responsible for higher methane production, even by a small amount, significantly increased methane production. If the lower methane production associated with *Calluna vulgaris* was due to the presence of condensed tannins (Frutos *et al.*, 2002), it would be expected that the opposite would be the case (i.e. that a slight increase in the proportion of *Calluna vulgaris* in the mixture would significantly reduce methane production). This was not, however, the case and there was therefore no evidence from this study that condensed tannins present in *Calluna vulgaris* were responsible for the lower methane production from this species. Although *Calluna vulgaris* has been shown to have a high condensed tannin content (Frutos *et al.*, 2002), the concentration of condensed tannins in the sample used in this experiment was not known. However, assuming that the expected high concentration of condensed tannins in the *Calluna vulgaris* was present, the results of this experiment do not suggest that condensed tannins have the expected effect of reducing methane production *in vitro*, as demonstrated in previous publications (Animut *et al.*, 2008; Frutos *et al.*, 2002; Tavendale *et al.*, 2005).

Differences between *Festuca spp.* and *Calluna vulgaris* in terms of digestibility were likely to be the cause of some differences in their respective methane production potentials. When methane production parameters were not corrected for digestibility,

there were greater differences between the mixtures of the plant species. Without correction for digestibility, there was also a linear relationship between the proportion of *Festuca spp.* in the mixture and methane production at 36 hours.

### **6.5.5 Conclusions**

The addition of just a small amount of *Festuca spp.* to the substrate mixture was found to significantly increase methane production potentials of feed samples. Therefore, there is little evidence that condensed tannins, assumed to be present in high concentrations in *Calluna vulgaris* samples, affected the methane production potentials of substrate mixtures. Changes in methane production may have been caused by differences between the digestibilities of the *Festuca spp.* and *Calluna vulgaris* samples: these samples were selected for this experiment partially due to the contrast between their digestibilities shown in Section 6.3. The results of this experiment suggest that differences in methane production between these samples were likely to be caused by a combination of factors, including differences in digestibility as well as other compositional characteristics of the two plant species.

## **6.6 *In vitro* gas production to compare a variety of upland plants, with and without the addition of polyethylene glycol (PEG)**

### **6.6.1 Introduction**

The previous study (Section 6.5) suggested that there was no effect of condensed tannins, present in *Calluna vulgaris*, on the *in vitro* methane production. The aim of this study was to investigate this further. Samples of forbs (*Calluna vulgaris* and *Vaccinium myrtillus*), which contain relatively high concentrations of condensed tannins (Frutos *et al.*, 2002), and grasses (*Festuca spp.* and *Molinia caerulea*), which contain relatively low condensed tannins (Montossi *et al.*, 1997), were therefore used in the experiment discussed in this section. Condensed tannins were not evaluated for the samples used in this experiment due to time constraints.

Polyethylene glycol (PEG) suppresses the effects of condensed tannins by reacting preferentially with them, preventing the formation of tannin-protein complexes (Priolo *et al.*, 2000). If PEG is present in sufficient quantity, it reduces or removes the effects of condensed tannins, which include lessening methane emissions from rumen fermentation (Waghorn, 2008). Therefore, by adding PEG to gas production samples, it was possible to determine whether there was an effect of condensed tannins on the methane production of plants known to contain these tannins. Previous studies have found that there were significant increases in both gas production and digestibility when PEG was added to samples containing condensed tannins (Arhab *et al.*, 2009; Basha *et al.*, 2013).

## **6.6.2 Materials and methods**

### **6.6.2.1 Samples used**

The samples used in this experiment were numbers 57, 58, 61 and 63 in Table 14.

These were samples of *Calluna vulgaris*, *Vaccinium myrtillis*, *Molinia caerulea* and *Festuca spp.*, collected in August 2011. A standard grass silage sample (Section 6.1.4.2) and a blank sample, containing no substrate, were also used. Each sample was used in triplicate, both with and without PEG.

### **6.6.2.2 PEG**

The PEG used had a molecular weight of 3350. One gram of PEG was used in each sample.

### **6.6.2.3 Rumen Inoculum**

For this experiment, two of the fistulated cows used in the previous experiments had died. Therefore, it was only possible to take rumen fluid from the one remaining fistulated cow, which may have implications for the results.

### **6.6.2.4 Method**

The method used is described in detail in Section 6.2. One gram of PEG (molecular weight 3350) was added to bottles containing PEG at the same stage as adding the substrates.

### **6.6.2.5 Calculations and model fitting**

Cumulative volumes of methane per gram of dry matter digested were calculated using the percentages of methane in gas produced, total volumes of gas at each time point and the estimates of digestibility for each sample. The results were also corrected for blanks. The calculations are detailed in Section 6.2.6. The France *et al.*

(1993) model was fitted to the as explained in Section 6.2.7. Standard errors were also calculated.

#### **6.6.2.6 Statistical analysis**

ANOVA was used to determine whether there were significant differences between species and between samples with and without PEG in terms of total methane production in the system, fractional rate of degradation, and cumulative methane production at 16, 24, 36 and 48 hours. The ANOVA treatment in GenStat 16th edition (2013) was PEG\*Species. This showed whether there were significant differences between species and between samples with and without PEG, as well as determining whether there were any significant interactions between presence of PEG and species.

## 6.6.3 Results

### 6.6.3.1 Methane production curves

Figure 36 shows the methane production curves for the five different substrates (including the standard silage sample), with and without the addition of PEG. In all samples, excepting *Molinia caerulea* and *Festuca spp.*, the addition of PEG increased methane production by samples.

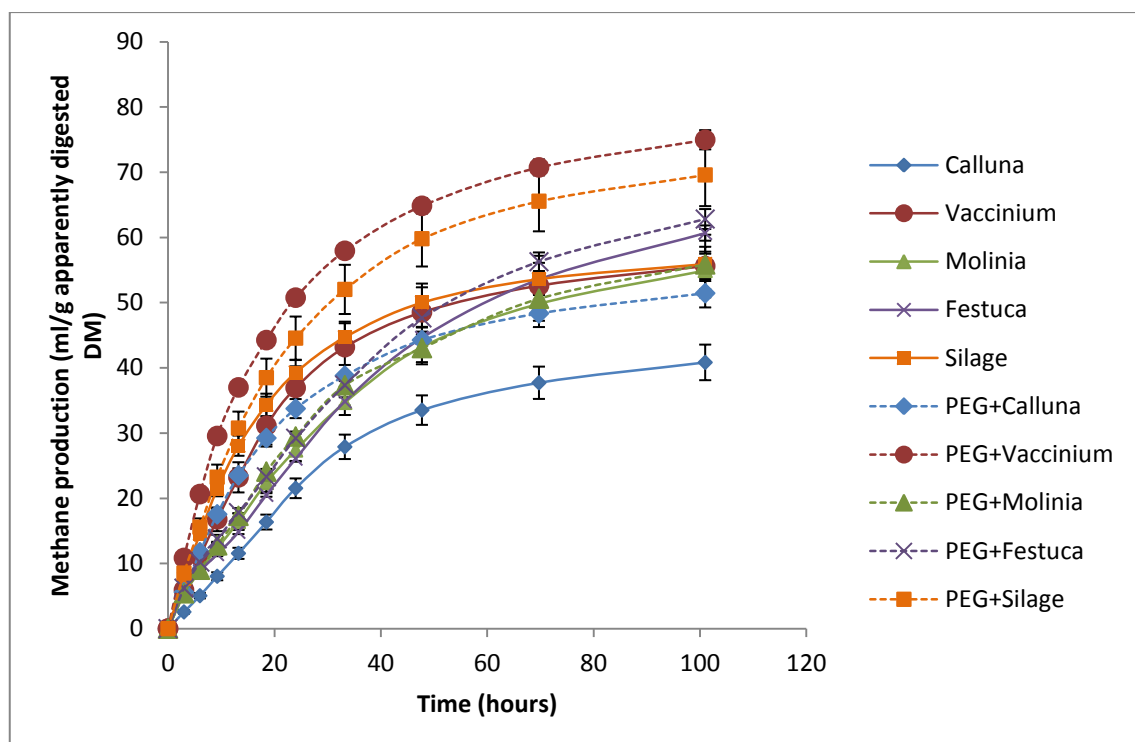


Figure 36: Methane production curves with and without the addition of PEG

### 6.6.3.2 ANOVA

Modelled values for total methane production in the system, fractional rate of degradation, and methane production at 16, 24, 36 and 48 hours are shown in Table 22. With regard to total methane production in the system, and methane produced at 16, 24, 36 and 48 hours, there were significant differences between plant species and between those samples that contained PEG and those that did not. There was also a significant interaction between species and the addition of PEG regarding total

methane produced in the system ( $P=0.021$ ) and methane produced at 16 hours ( $P=0.035$ ). However, no significant interactions were observed in methane produced at 24 ( $P=0.062$ ), 36 ( $P=0.082$ ) and 48 ( $P=0.075$ ) hours. There were significant differences between species and presence of PEG in terms of fractional rates of degradation. There were also significant interactions ( $P<0.001$ ) between species and presence of PEG in terms of these parameters. The interactions between the presence or absence of PEG and the species suggest that the effect of adding PEG to gas production samples affects methane production by some species more than others. This is also evident in Figure 36: the effect of adding PEG to the gas production profiles of *Molinia caerulea* and *Festuca spp.* is minimal in comparison to the effect on the profiles *Calluna vulgaris*, *Vaccinium myrtillis*, and the standard silage sample.



**Table 22: Modelled methane production parameters for species with and without PEG**

Methane production parameter	Species means					SED (species)	p (species)
	<i>Calluna vulgaris</i>	<i>Festuca spp.</i>	<i>Molinia caerulea</i>	<i>Vaccinium myrtillis</i>	Standard silage sample		
Total methane (ml/g apparently digested DM)						3.00	<0.001
Without PEG	43.5	71.0	60.3	56.7	56.9		
With PEG	52.4	71.9	59.8	76.1	68.0		
SED (PEG)	1.90						
p (PEG)	<0.001						
Fractional rate of degradation (g/h)						0.00	<0.001
Without PEG	0.038	0.021	0.027	0.050	0.051		
With PEG	0.045	0.022	0.030	0.042	0.058		
SED (PEG)	0.00059						
p (PEG)	0.002						
Methane at 16 hours (ml/g apparently digested DM)						1.44	<0.001
Without PEG	20.5	20.7	21.5	31.7	35.6		
With PEG	26.9	21.5	23.0	35.8	41.3		
SED (PEG)	0.91						
p (PEG)	<0.001						
Methane at 24 hours (ml/g apparently digested DM)						1.85	<0.001
Without PEG	26.7	28.6	29.1	40.0	40.0		
With PEG	34.6	29.8	31.0	46.7	51.3		
SED (PEG)	1.17						
p (PEG)	<0.001						
Methane at 36 hours (ml/g apparently digested DM)						2.24	<0.001
Without PEG	33.1	38.3	37.9	47.7	47.7		
With PEG	42.0	39.6	39.8	57.8	59.7		
SED (PEG)	1.42						
p (PEG)	<0.001						
Methane at 48 hours (ml/g apparently digested DM)						2.48	<0.001
Without PEG	37.0	45.7	44.2	51.8	51.8		
With PEG	46.4	47.2	45.9	64.7	63.9		
SED (PEG)	1.57						
p (PEG)	<0.001						

#### **6.6.4 Discussion**

The significant increases in the methane production parameters associated with the addition of PEG was expected for samples containing large quantities of condensed tannins; *Calluna vulgaris* and *Vaccinium myrtillis*. For samples that probably did not contain condensed tannins in any large quantity, *Molinia caerulea* and *Festuca spp.*, there was little effect of PEG on the methane production parameters. This is in agreement with Tiemann *et al.* (2008), who found that adding PEG to tanniferous legumes increased the fermentation parameters, but that the addition of PEG to non-tanniferous legumes had no effect.

It was not expected that there would be a significant effect of adding PEG to the standard silage sample, as this was not expected to be a tanniferous sample. It is not clear why this effect occurred: tannins have been used as additives in silage production and have been shown to reduce the proteolysis that occurs during the ensiling process (Tabacco *et al.*, 2006). However, in this case, condensed tannins were not used in the ensiling process. This suggests that PEG may interact with other components of feed material as well as condensed tannins.

#### **6.6.5 Conclusion**

It is not possible to state conclusively that PEG increased methane production parameters in some species due to binding with condensed tannins, although this is suggested by the results.

## Chapter 7

Validation of the *in vitro* gas production technique by comparison of gas production profiles of feed samples from animals in methane chambers with methane chamber results

## **7 Validation of the *in vitro* gas production technique by comparison of gas production profiles of feed samples from animals in methane chambers with methane chamber results**

### **7.1 Introduction**

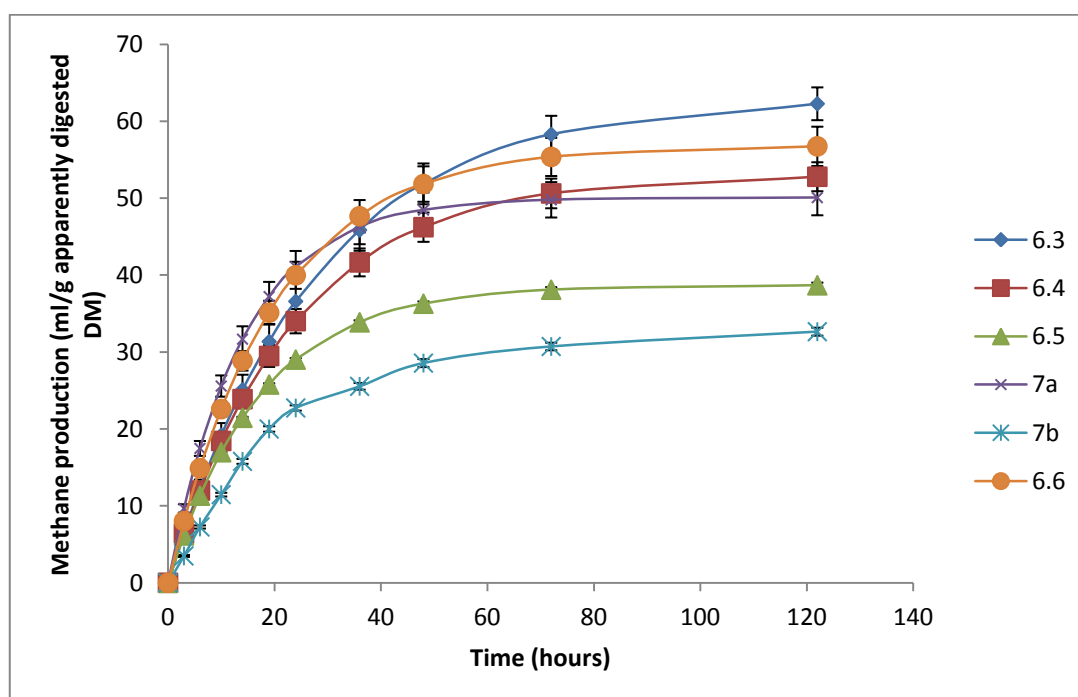
The *in vitro* gas production technique could provide a quick and simple means of estimating methane emissions by sheep based on the methane production potential of the feeds offered and intake measurements or estimates of feed intake. Previous studies have used the *in vitro* gas production technique to evaluate feed characteristics, such as digestibility (Blümmel and Ørskov, 1993; Brown *et al.*, 2002). However, there is a lack of published data regarding the use of the technique specifically used to predict *in vivo* methane output by sheep.

#### **7.1.1 Aims and objectives**

In Chapter 6, Sections 6.3 to 6.6, the potential of the *in vitro* gas production technique to differentiate between upland plant samples in terms of fractional rate of degradation, total methane potential in the system and cumulative methane production at various time intervals to represent potential rumen retention times. The aim of this section is to validate the use of the technique as a proxy for methane potential of feeds by comparison of gas production data from feed samples, which have been fed to animals during methane chamber experiments, with the methane chamber data from these animals.

### 7.1.2 Necessity to correct for standard silage sample

As explained in Chapter 6, Section 6.1.2, the most significant cause of error in the technique is due to variability of rumen inoculum, even when taken from the same animals, fed the same diet and at the same time of day. As in Chapter 6, Section 6.6, there was only one fistulated cow available for this experiment. Due to methodological constraints and a large number of samples, it was not possible to complete the gas production analysis using just one batch of rumen fluid. Statistical analysis (repeated measures ANOVA, performed in Genstat 16th edition (2013)) was used to compare the methane production curves for the standard silage sample (Chapter 6, Section 6.1.4.2), which was used in all gas production experiments. Figure 37 shows the methane production curves for the standard silage sample used in each experiment (the numbers 6.3–6.6 relate to experimental sections in this Chapter 6; 7a and 7b relate to the two different experimental sections within this chapter).



**Figure 37: Methane production curves for the standard silage sample in different gas production experiments**

Figure 37 clearly shows the variation between experimental runs, and statistical analysis using ANOVA in GenStat 16th edition (2013) showed significant ( $P < 0.001$ ) differences between standards in terms of total methane production in the system, fractional rate of degradation, and cumulative gas production at 16, 24, 36 and 48 hours. It was, therefore, essential to correct for the differences between standard samples when comparing between experimental runs using different batches of rumen inoculum. The method used for correction is explained in Section 7.2.5.

## 7.2 Materials and Methods

### 7.2.1 Samples used

#### 7.2.1.1 Feed samples from methane chamber experiments

Samples of feeds offered to sheep were collected during four different methane chamber experiments (see Chapter 2). These feeds were perennial ryegrass, permanent pasture (containing a variety of grass species [e.g. perennial and Italian ryegrass, cocksfoot, and timothy], clovers, and some weeds), grass nuts, and *Molinia caerulea*. Perennial ryegrass, permanent pasture, and *Molinia caerulea* were offered using zero grazing. Plants were cut using a Haldrup Harvester in IBERS fields (perennial ryegrass and permanent pasture) or at Pwllpeiran, an upland site (*Molinia caerulea*). Plants were harvested daily or as required and stored in a large walk-in refrigerator next to the sheep shed. Grass nuts were also kept in this refrigerator during the grass nuts experiment.

These experiments each used 32 sheep of four different breeds (Welsh mountain, Scottish blackface, Welsh mule, and Texel). The majority of the sheep remained the same throughout the four experiments, though there were some changes. During the experiments, sheep were fed either perennial ryegrass, permanent pasture, grass nuts, or *Molinia caerulea*. Four sheep entered the chambers for three days at a time. There were, therefore, eight runs of sheep being put through chambers. Samples of feed offered were taken on a daily basis and bulked for each three day period so that there was a feed sample per run of sheep entering the chambers. This meant that there was a total of 32 samples, eight samples of each of the feeds offered, and methane emission data from the sheep that consumed these feeds. These samples were used along with the standard silage sample (Chapter 6, Section 6.1.4.2) and blanks. All

samples were analysed in triplicate. Due to the large number of replicates, half of the samples were analysed at once and the standard sample and blanks were analysed with each half of the feed samples.

### **7.2.2 Animals**

Thirty-two mature, barren ewes of four different breeds (Welsh mountain, Scottish blackface, Welsh mule and Texel) were put in methane chambers for three day periods as described in Chapter 2 (Section 2.2). The sheep were adapted to and fed on each of the four different feeds during four different methane chamber experiments. The sheep were put in chambers in groups of four, so methane chamber measurements for eight groups of four sheep were collected during each experiment. Samples of feed offered were taken for each group of sheep whilst in chambers.

### **7.2.3 Method**

The method used is described in detail in Chapter 6, Section 6.2.

### **7.2.4 Calculations and model fitting**

Cumulative volumes of methane per gram of dry matter digested were calculated using the percentages of methane in gas produced, total volumes of gas at each time point and the estimates of digestibility for each sample. The results were also corrected for blanks. The calculations are detailed in Chapter 6, Section 6.2.6. The France *et al.* (1993) model was fitted to the data as explained in Chapter 6, Section 6.2.7. Standard errors were also calculated.



### 7.2.5 Correction for standard samples

Having fitted the France *et al.* (1993) model to the data, it was clear that correction for standard samples was necessary, as discussed in Section 7.1.2. In the first experimental run, which tested perennial ryegrass and permanent pasture, the standard sample produced significantly more methane and had a significantly higher fractional rate of degradation than in the second experimental run, which analysed grass nuts and *Molinia caerulea*.

Because the data includes rates, correcting the data to account for standards was not simply a case of scaling up the total gas production potentials in the system as the total would not account for differences in rates of gas production. Therefore, the France *et al.* (1993) model was fitted to the data from the first experimental run. The model parameters were used to calculate the methane produced at each of the measurement timepoints (3t, 6t, 122t...) from the second experimental run. Correction factors, calculated using the standard sample data from both experimental runs, were multiplied by the raw data from the second experimental run in order to scale this data to that of the first experimental run.

Correction factors were calculated to scale the standards from the second experimental run to the standards from the first experiments. These were calculated by dividing the values from the first experimental by the values from the second experimental run. This gave correction factors that were multiplied by the second experimental run values to scale them to the values from the first experimental run.

Once the correction factors were applied to the raw data from the second experimental run, the France *et al.* (1993) model was fitted to the corrected data.

### **7.2.6 Statistical analysis: ANOVA**

Repeated measures ANOVA in GenStat 16th edition (2013) was used to compare the different feeds and samples in terms of total methane production potential in the system, fractional rate of degradation, and cumulative methane production at 16, 24, 36, and 48 hours.

### **7.2.7 Statistical analysis: Regression**

The dry matter (DM) intakes of the sheep during chamber experiments was used to calculate predicted emissions from sheep, based on the *in vitro* gas production data for the different feeds. The methane production values used were the total methane production, and the cumulative methane productions at 16, 24, 36, and 48 hours.

Functional bisector regression in GenStat 16th edition (2013) was used to correlate the predictions made using the gas production and DM intake data with the methane emissions by each sheep as calculated from the methane chamber experiments.

## 7.3 Results

### 7.3.1 Methane production curves

Figure 38 shows the average methane production curves, corrected for digestibility, for each of the different types of feed, the grass nuts and *Molinia caerulea* results being scaled up to those of the perennial ryegrass and permanent pasture using the method described in Section 7.2.5. Standard error bars are shown, although some are too small to be visible. Figure 39 shows average methane production curves for the different feeds, uncorrected for digestibility, but corrected according to differences between standard silage samples (as above).

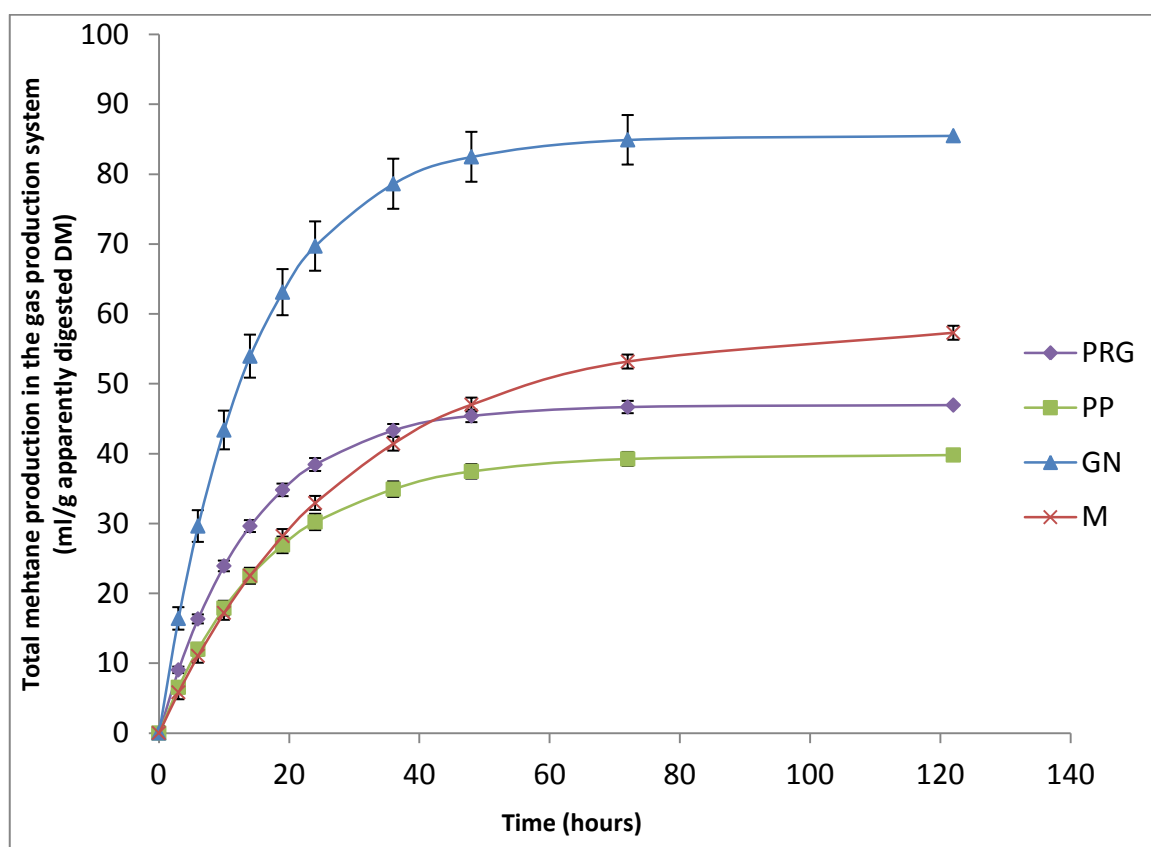


Figure 38: Methane production curves corrected for digestibility (PRG=perennial ryegrass, PP=permanent pasture, GN=grass nuts, M=*Molinia caerulea*)

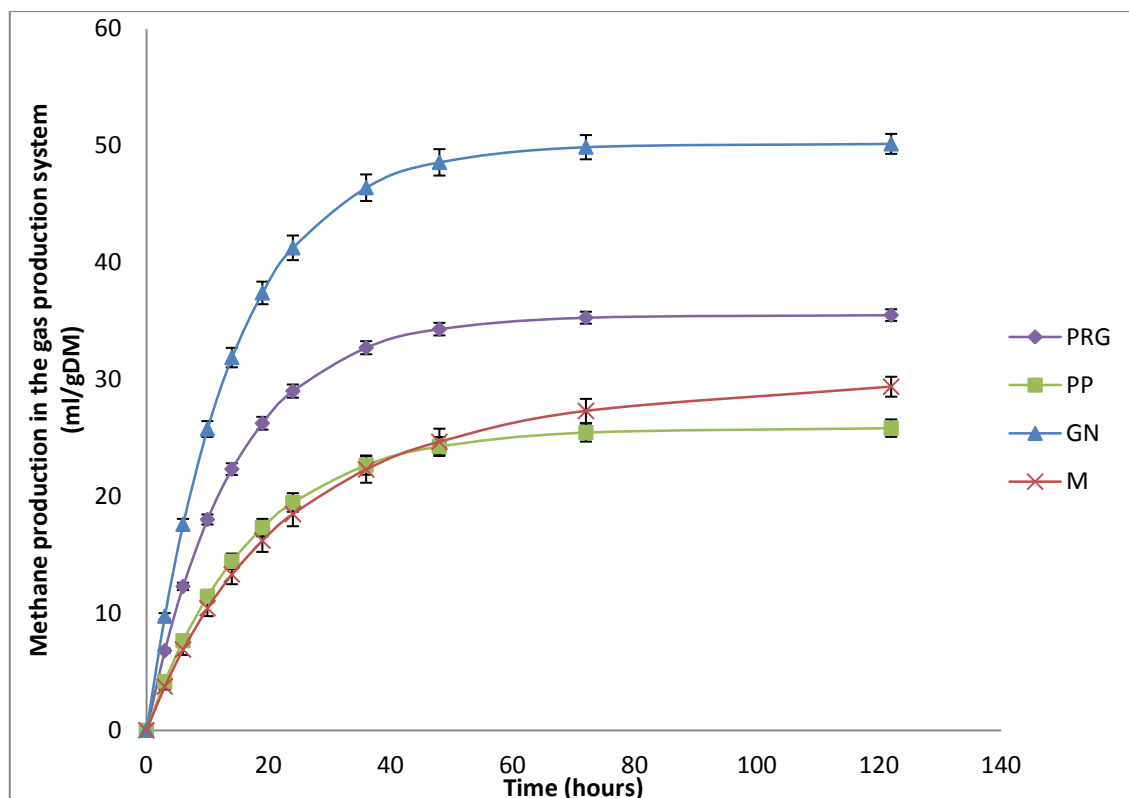


Figure 39: Methane production curves uncorrected for digestibility (PRG=perennial ryegrass, PP=permanent pasture, GN=grass nuts, M=*Molinia caerulea*)

### 7.3.2 Repeated measures ANOVA

There were significant ( $P < 0.01$ ) differences between different feed materials in terms of total methane production in the system, fractional rate of degradation, and cumulative methane production at 16, 24, 36 and 48 hours. The table of means for these parameters, and the significant differences between them, are shown in Table 23. The superscript letters denote significant differences between species at a one percent level. Grass nuts and perennial ryegrass samples degraded at a significantly faster rate ( $P < 0.01$ ) than permanent pasture, which degraded at a significantly faster rate ( $P < 0.01$ ) than *Molinia caerulea*. Grass nuts samples produced significantly more methane ( $P < 0.01$ ) than all of the other feeds at 16, 24, 36 and 48 hours and in terms of total methane production in the system. Similar results were observed using data not

corrected for digestibility (Table 24), although perennial ryegrass samples produced significantly more methane than *Molinia caerulea* in this case ( $P<0.01$ ).

**Table 23: Means total methane (ml/g apparently digested DM), fractional rate of degradation (g/h), and methane produced at 16, 24, 36 and 48 hours (ml/g apparently digested DM) for the feeds tested using the gas production technique using data corrected for digestibility**

Methane production parameter	Feed means				S.E.D	P
	Perennial ryegrass	Permanent pasture	Grass nuts	Molinia		
Total methane (ml/g apparently digested DM)	47.0 <sup>a</sup>	39.8 <sup>a</sup>	85.5 <sup>c</sup>	58.4 <sup>b</sup>	2.78	<0.001
Fractional rate of degradation (g/h)	0.066 <sup>c</sup>	0.054 <sup>b</sup>	0.068 <sup>c</sup>	0.034 <sup>a</sup>	0.0025	<0.001
Methane at 16 hours (ml/g apparently digested DM)	32.0 <sup>b</sup>	24.4 <sup>a</sup>	57.9 <sup>c</sup>	24.9 <sup>a</sup>	2.45	<0.001
Methane at 24 hours (ml/g apparently digested DM)	38.5 <sup>b</sup>	30.2 <sup>a</sup>	69.7 <sup>c</sup>	32.9 <sup>ab</sup>	2.68	<0.001
Methane at 36 hours (ml/g apparently digested DM)	43.4 <sup>b</sup>	35.1 <sup>a</sup>	78.6 <sup>c</sup>	41.4 <sup>ab</sup>	2.83	<0.001
Methane at 48 hours (ml/g apparently digested DM)	45.4 <sup>b</sup>	37.4 <sup>a</sup>	82.5 <sup>c</sup>	47.0 <sup>b</sup>	2.84	<0.001

Superscript letters denote significant differences ( $P<0.01$ ) between feed samples.

**Table 24: Means total methane (ml/g DM), fractional rate of degradation (g/h), and methane produced at 16, 24, 36 and 48 hours (ml/g DM) for the feeds tested using the gas production technique using data uncorrected for digestibility**

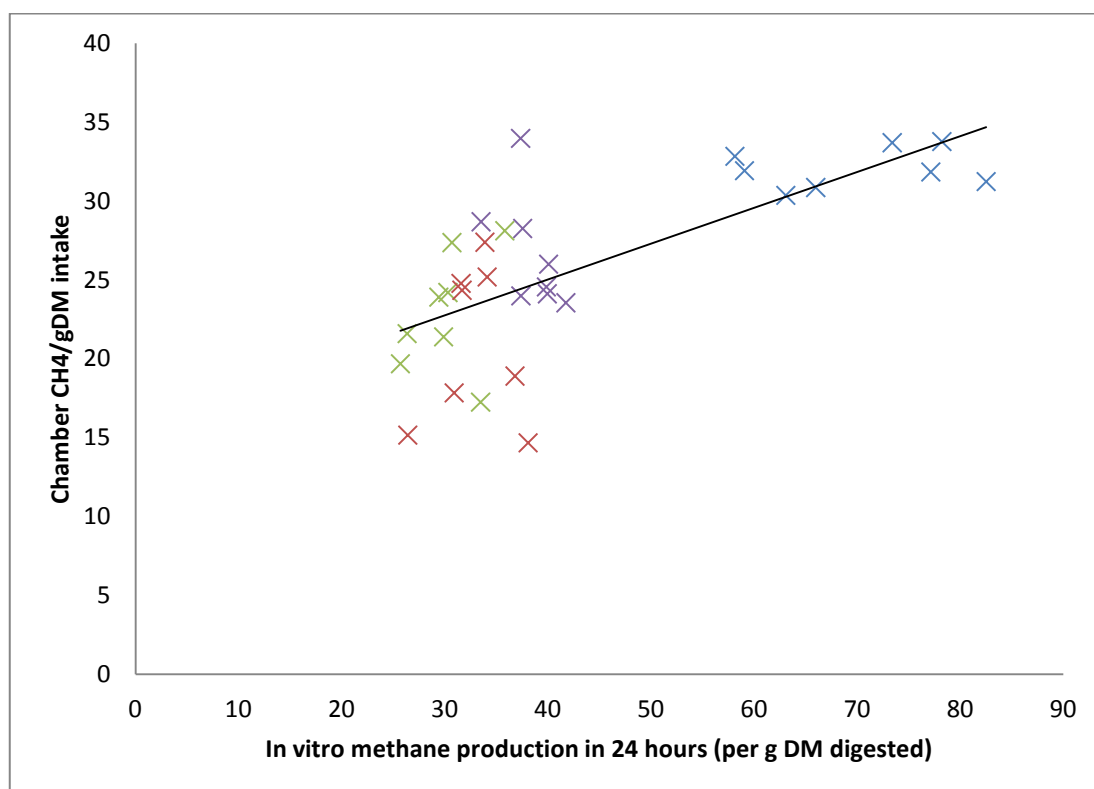
Methane production parameter	Feed means				S.E.D	P
	Perennial ryegrass	Permanent pasture	Grass nuts	Molinia		
Total methane (ml/g DM)	35.5 <sup>c</sup>	25.8 <sup>a</sup>	50.2 <sup>d</sup>	29.4 <sup>b</sup>	1.08	<0.001
Fractional rate of degradation (g/h)	0.066 <sup>c</sup>	0.053 <sup>b</sup>	0.068 <sup>c</sup>	0.041 <sup>a</sup>	0.002	<0.001
Methane at 16 hours (ml/g DM)	24.1 <sup>b</sup>	15.7 <sup>a</sup>	34.3 <sup>c</sup>	14.6 <sup>a</sup>	1.06	<0.001
Methane at 24 hours (ml/g DM)	29.0 <sup>b</sup>	19.5 <sup>a</sup>	41.3 <sup>c</sup>	18.5 <sup>a</sup>	1.21	<0.001
Methane at 36 hours (ml/g DM)	32.7 <sup>b</sup>	22.7 <sup>a</sup>	46.4 <sup>c</sup>	22.3 <sup>a</sup>	1.28	<0.001
Methane at 48 hours (ml/g DM)	34.3 <sup>b</sup>	24.3 <sup>a</sup>	48.6 <sup>c</sup>	24.7 <sup>a</sup>	1.26	<0.001

Superscript letters denote significant differences ( $P<0.01$ ) between feed samples.

### 7.3.3 Regression between gas production data and methane chamber daily methane emissions

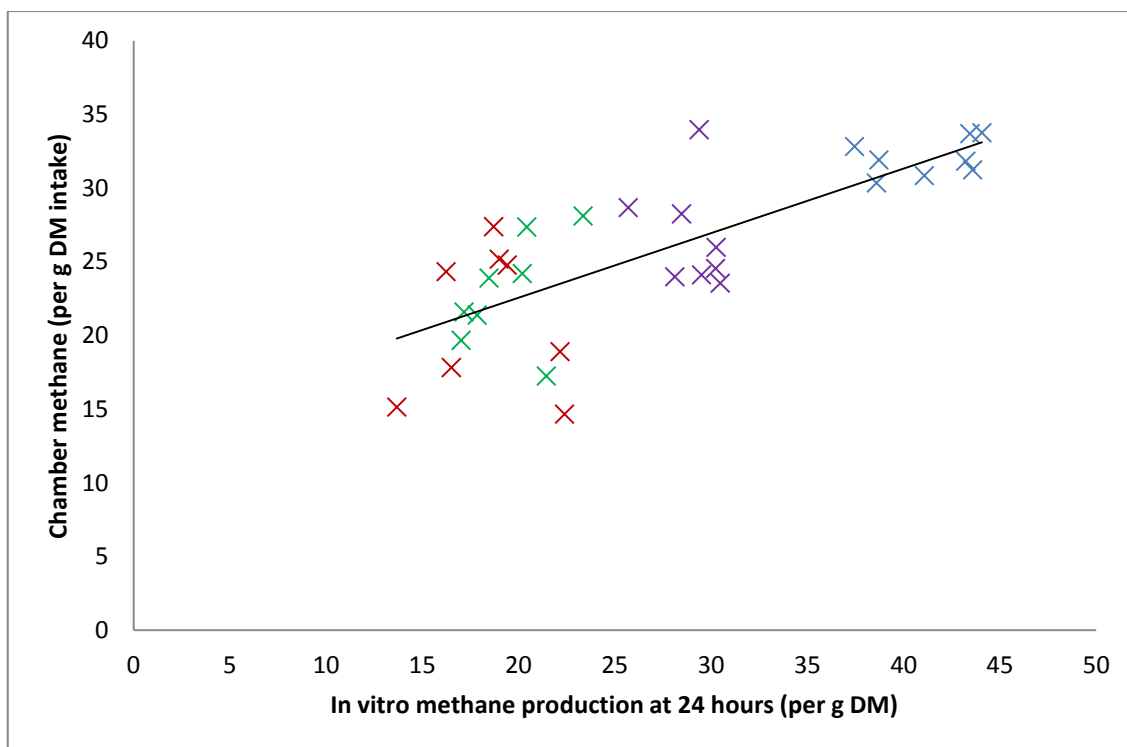
There was a significant positive correlation ( $y=10.3(\text{s.e. } 2.52)+0.35(\text{s.e. } 0.058)x$ ) ( $R=0.68$ ;  $P<0.001$ ) between *in vitro* methane production per gram of DM digested in 24 hours and daily chamber methane emissions by sheep per gram of DM intake, suggesting that *in vitro* gas production data can be used to predict methane emissions provided that the DM intake, or an estimate of the DM intake is known. The correlation coefficient was 0.46. The correlation is shown in Figure 40.

There was also a significant positive correlation ( $y=13.8(\text{s.e. } 1.85)+0.44(\text{s.e. } 0.051)x$ ) ( $R=0.77$ ;  $P<0.001$ ) between *in vitro* methane production per gram of DM in 24 hours (uncorrected for digestibility) and daily chamber methane emissions by sheep per gram of DM intake (Figure 41).



**Figure 40: Functional bisector relationship between *in vitro* gas production at 24 hours (per g DM digested) and daily methane emissions per gram of DM intake**

Red=*Molinia caerulea*; Green=permanent pasture; Purple=perennial ryegrass; Blue=grass nuts.



**Figure 41: Functional bisector relationship between *in vitro* methane production (per g DM, uncorrected for digestibility) and chamber methane (per g DM intake)**

Red=*Molinia caerulea*; Green=permanent pasture; Purple=perennial ryegrass; Blue=grass nuts.

## 7.4 Discussion

### 7.4.1 ANOVA

Grass nuts and perennial ryegrass degraded at a faster rate than permanent pasture, which degraded more quickly than *Molinia caerulea*. These differences were probably associated with the NDF content of the feeds, with those containing higher NDF percentages being less readily degradable. In terms of the total methane production in the system, grass nuts produced significantly more methane than all of the other feeds, and *Molinia caerulea* produced more methane than perennial ryegrass and permanent pasture. However, methane production in the gas production system may not translate directly to methane production potential of feeds when digested by an animal. Rumen fermentation can only occur while the feed is present in the rumen; the rumen retention time of feeds will have an impact on how much methane is produced. Typical rumen retention times vary, and are higher when the feed is more fibrous (Menke *et al.*, 1979). Twenty four hours could be considered a typical rumen retention time, so it may be more appropriate to compare feeds in terms of methane production at 24 hours rather than total methane production in the system. In terms of methane produced at 24 hours, grass nuts were significantly higher than the other feeds. Perennial ryegrass produced more methane at 24 hours than *Molinia caerulea* and permanent pasture, though the difference between perennial ryegrass and *Molinia caerulea* was non-significant.

### 7.4.2 Functional bisector regression

The significant relationship between *in vitro* methane production (both ml/g apparently digested DM and ml/g DM) at 24 hours and mean methane produced by



sheep fed on the same samples, as measured using methane chambers, suggests that the technique could potentially be used to predict methane output by sheep, provided that DM intake is known, or can be reasonably estimated. The correlation coefficient was not particularly high when *in vitro* methane production per gram of apparently digested DM was used, and slightly higher when *in vitro* methane production per gram of DM was used. It may be possible to improve the fit to the correlation by altering the time taken to be the rumen retention time for different feeds; this is discussed in Section 7.4.3.

### **7.4.3 The importance of rumen retention times**

It is likely that the feeds analysed using the gas production technique all have different average rumen retention times. If the retention times were known for each feed, it would be possible to take methane production at different times, depending on the feed offered, as estimates of methane production per gram of DM intake. Rumen retention times depend on a variety of factor, including feeding level, particle size and dietary composition (Sriskandarajah *et al.*, 1981). Feeding level is something that is easily measured in intensive systems, though it is more complicated for grazing animals, as is dietary composition. The feeds were all ground to the same particle size in the gas production experiments. Measuring rumen retention times is possible using dietary markers; however, this is too complicated to do at a large on-farm scale. It may be possible to introduce correction factors for rumen retention times, based on NDF content of feed and feeding level. Further work should focus on this.

## 7.5 Conclusions and recommendations

A number of conclusions and recommendations for the direction of further research on the use of the *in vitro* gas production technique to predict methane emissions by sheep can be drawn from the results of experiments discussed in Chapter 6 and this chapter.

- The *in vitro* gas production technique can differentiate between different upland plant samples.
- Samples containing a higher percentage of NDF produced more methane per gram of DM *in vitro* (when corrected for digestibility and when not corrected for digestibility), which was to be expected. There was no relationship between methane produced *in vitro* with ADF or WSC content of samples.
- The France *et al.* (1993) model may not fit well to methane production from plant material that is senescent.
- There is conflicting evidence as to whether the presence of condensed tannins in plant materials decreases the methane produced *in vitro* by the samples.
- Methane production measured *in vitro* from feeds can be used to predict, to an extent, *in vivo* methane production by sheep fed on these feeds.
- The *in vitro* gas production technique, therefore, could provide a quick and simple means of testing animal feeds for their *in vivo* methane potentials.
- Further work should focus on developing correction factors based on rumen retention times, as discussed in Section 7.4.3.

## Chapter 8

The use of Fourier-transform infrared (FTIR) spectroscopy to detect differences between plant samples that may be associated with their methane potentials

## **8 The use of Fourier-transform infrared (FTIR) spectroscopy to detect differences between plant samples that may be associated with their methane potentials**

### **8.1 Introduction**

Spectroscopy techniques, such as near infrared reflectance (NIR) spectroscopy can provide a rapid and inexpensive means of estimating the chemical composition of animal feeds (Poppi, 1996), such as crude protein (CP) and NDF concentrations (Landau *et al.*, 2006). Consequently, NIR spectroscopy is widely used in for quality control and assurance purposes in the animal feed industry (Graham *et al.*, 2013). It can also be used to accurately predict *in vitro* and *in vivo* digestibilities of feeds, as well as the concentration of tannins in a wide range of browse species (Landau *et al.*, 2006).

Fourier-transform infrared (FTIR) spectroscopy is a similar rapid-throughput technique, which can also be used to obtain information about the chemical composition of feed samples with varying accuracy depending on feed type (Belanche *et al.* 2013). A recent study found that models based on FTIR data from 80 different feeds provided relatively poor estimation of DM degradation parameters as measured using an *in situ* technique (Belanche *et al.*, 2014). However, FTIR has been used to successfully detect differences between faecal samples of sheep fed on different diets (Moorby *et al.*, 2010; Parveen *et al.*, 2008). As it is possible to distinguish between diets using FTIR and diet is known to affect enteric methane production in ruminants, it may be possible to use FTIR to distinguish between diets in terms of their methane potentials.

Furthermore, NIR spectroscopy has been shown to accurately predict the intake potential of silage by cattle (Steen *et al.*, 1998). FTIR spectroscopy could potentially also be used to predict characteristics of feeds that affect feed intake, thereby predicting methane production, as feed intake is one of the main factors driving methane production by ruminants (Molano and Clark, 2008). If spectroscopy techniques can be used to predict intake, they could also potentially predict methane production potentials of feeds. Moreover, Moss and Givens (2000) found that NIR spectroscopy was a good predictor for ruminant methane emissions; this suggests that FTIR spectroscopy could also be used for this purpose.

### **8.1.1 Aims and objectives**

The aims of the work described in this chapter were to establish whether FTIR spectra can be used to distinguish between plant and feed samples that have been shown to have different methane production potentials *in vivo* or *in vitro*, and whether FTIR can be used to predict. The objectives were as follows:

- To perform FTIR analysis on a number of samples that were fed to sheep in methane chambers (Chapter 2).
- To use principal component analysis (PCA) to determine whether there were distinct differences between feed samples associated with higher and lower methane emissions measured *in vivo*.
- To draw conclusions as to any differences found with regard to the causes of differences.

- To use partial least squares regression to determine whether the FTIR spectra could be used as a means of predicting methane output by sheep from which the feed samples were taken.

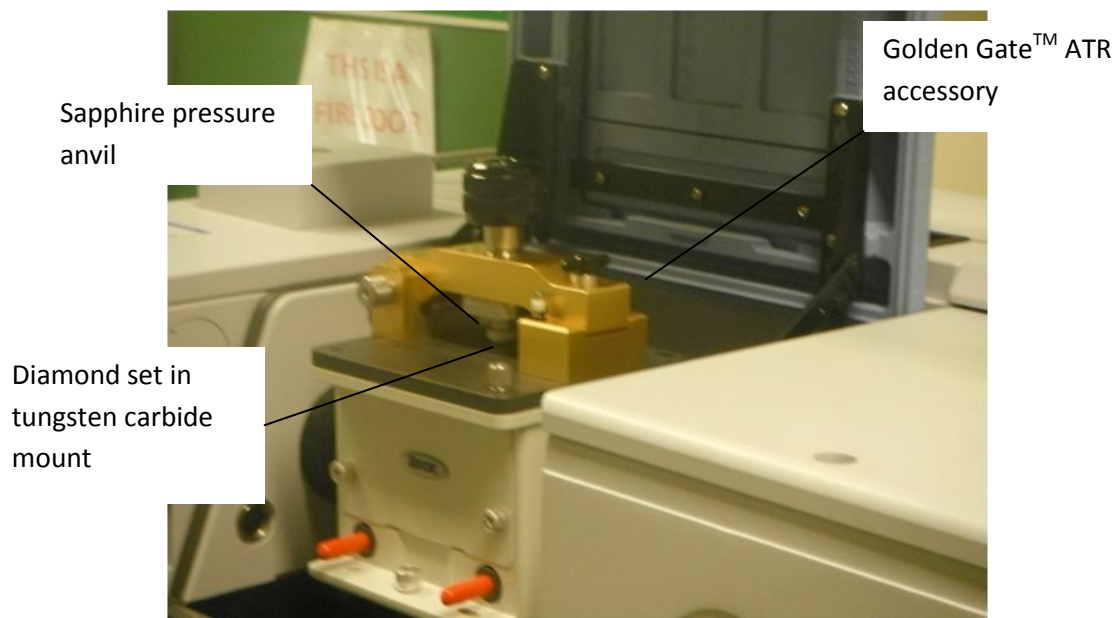
## 8.2 Materials and Methods

### 8.2.1 Samples used

The samples used in the experiment were the samples used in Chapter 7, which were those fed to animals during methane chamber experiments (Chapter 2). These consisted of eight samples each of grass nuts, perennial ryegrass, permanent pasture and *Molinia caerulea*, and one sample of silage. Average daily methane emissions from sheep from which the feed samples were taken were calculated and used as the observed values for the PLS regression of the FTIR data on methane chamber values.

### 8.2.2 FTIR spectrometer

The FTIR spectrometer used in the experiment was the Equinox 55 from Bruker UK Ltd., Coventry, UK fitted with a Golden Gate<sup>TM</sup> (Specac Ltd., Slough, UK) attenuated total reflectance (ATR) accessory (shown in Figure 42). This accessory uses a trapezoid crystal of type IIIa diamond. The sample, which was dried and ground, was pressed against the surface of the diamond using the inert sapphire pressure anvil. The IR beam was passed through the diamond using mirrors and hits the diamond at a very shallow angle. The IR beam was reflected within the diamond, which formed a wave that extended into the sample on the sample-covered surface of the diamond, before passing out of the other side to the detector. The spectrometer operates in a wavenumber range of 4000-600cm<sup>-1</sup>. The software used to collect the FTIR measurements was Opus (Bruker UK Ltd., Coventry, UK).



**Figure 42: FTIR spectrometer with Golden Gate™ accessory (closed)**

### **8.2.3 Measurement method**

The Golden Gate™ ATR accessory was fitted into the FTIR spectrometer. This signal was checked using the "Validate" menu, and the screws controlling the height of the accessory plate were adjusted to achieve a maximum amplitude (around 2000) and the peak position was saved. Bellows were then fitted either side of the accessory, between the accessory and the potassium bromide beamsplitters. The file pathway for saving the data collected was entered into the "Advanced" section of the "Advanced Measurements" option from the "Measure" menu.

A background measurement was taken by leaving the accessory open as shown in Figure 43. The "Measure/ Advanced Measurements/ Basic" menu was used, and the "Background Single Channel" option was selected. A background measurement was taken for every new sample.

The samples used were freeze dried and finely ground to pass through a 1mm sieve.

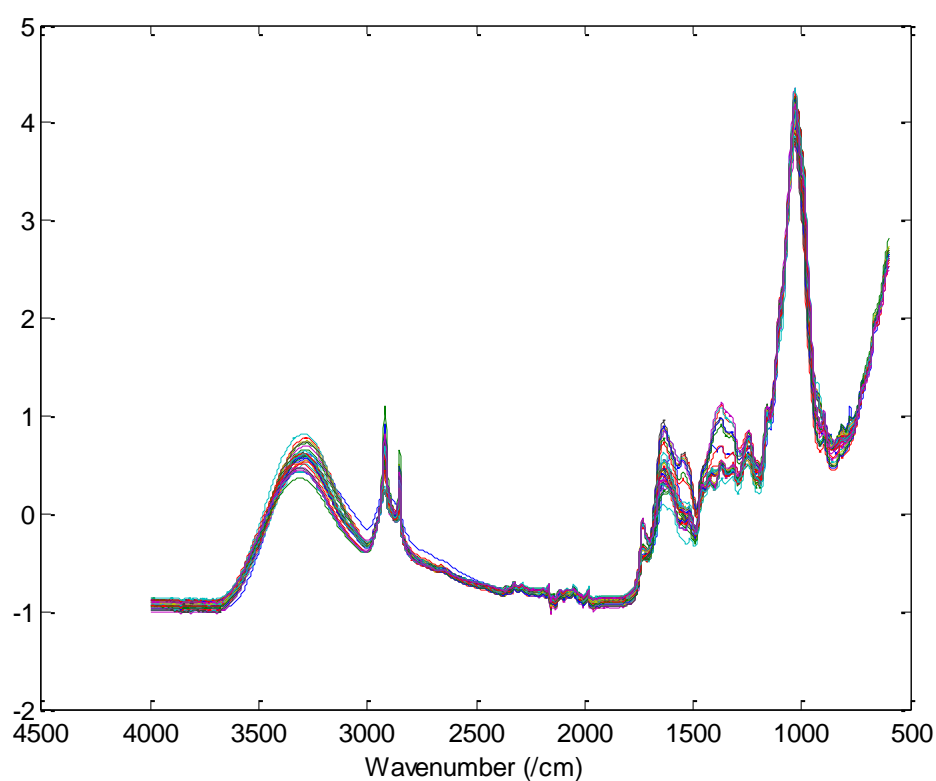
Using a spatula, a small amount of sample, enough to cover the exposed surface of the



diamond, was placed on the plate (see Figure 43). The accessory was then closed and the sapphire anvil was screwed down to crush the sample against the surface of the diamond. The "Measure/ Advanced Measurements/ Advanced" menu was used to input a file name consisting of the sample number. The "Measure/ Advanced Measurements/ Basic" was then used, and the "Sample Single Channel" option was selected. This produced a spectrum for the sample (Figure 44) and saved the data to the file pathway previously selected. The anvil was then unscrewed and the accessory opened. The sample was removed from the plate using a vacuum cleaner and replaced with another sample of the same plant/feed material, which was measured in the same way. For every few samples analysed, acetone was wiped over the plate to remove any sample remnants. All samples were analysed in triplicate.



**Figure 43: Golden Gate™ accessory whilst open, with a sample on the plate.**



**Figure 44: Example FTIR spectra from various plant samples. These data have been normalised to a mean absorbance of 0 and a standard deviation of 1 to account for differences in sample thickness.**

#### **8.2.4 Data conversion**

In order to analyse the data, it was necessary to convert the data to xy data. This was done by creating a new folder entitled 'xy data'. This was done using the 'Macro' menu in the Opus software.

#### **8.2.5 Data analysis**

Initially, principal components analysis (PCA) and partial least squares (PLS) regression was performed in GenStat 16th edition. However, GenStat was not particularly intuitive for these analyses and, to ensure that results were accurate, I received help from my supervisor (Dr. Jon Moorby) to conduct PCA, PLS regression and cross validation using Matlab (R2013a, MathWorks Inc., Cambridge, UK).

An initial visual check of the data was carried out to ensure that all spectra appeared normal. Any spectra that appeared to be odd were discarded. Mean spectra were calculated for each sample from the original data collected in triplicate; any spectra that appeared not to be normal were excluded from the mean values calculated. Absorbance spectra from individual samples varied in amplitude because of difference in the thickness of the samples analysed. To remove this component of variation from the dataset, all spectra were mean-centre normalised using the MAPSTD function of Matlab, which normalises data to a mean of zero and a standard deviation of 1. This mean-centred FTIR data was then used for subsequent analyses.

#### **8.2.5.1 Principal components analysis**

A large number of variables were identified using FTIR spectroscopy of feed samples. Principal components analysis was conducted in Matlab using the PCA function: this technique was used to simplify the data by replacing groups of variables with single variables (principal components). Each principal component identified was a linear combination of the original variables. The principal components with combined variances were equal to at least 80% of the total variance of the original data set were plotted. By examining the plotted data, it was possible to determine whether the FTIR data could be used to distinguish between the different feeds.

#### **8.2.5.2 Partial least squares regression**

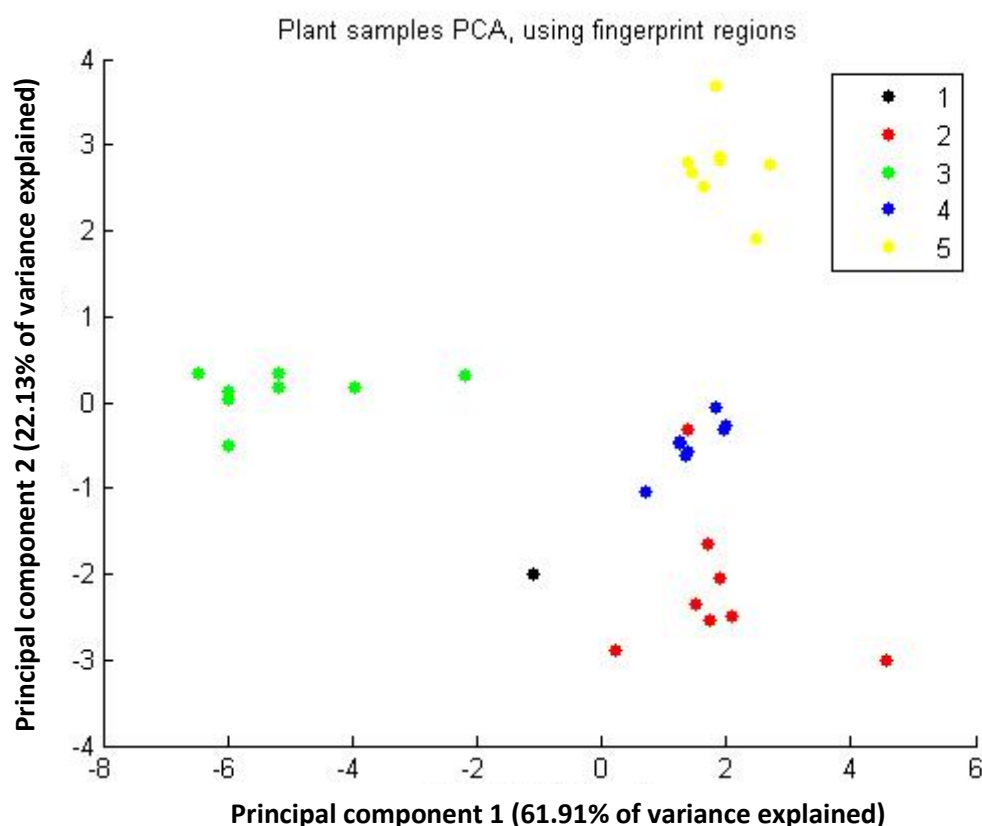
Partial least squares regression was also conducted in Matlab in order to determine whether the FTIR dataset from feeds offered to sheep could be used to predict methane output by sheep as measured in methane chambers. The PLS technique combines the principles of multiple linear regression and principal component

analysis, using correlated predictor variables (in this case from the FTIR dataset) to construct new predictor variables or "components", which are linear combinations of the original predictor variables. The components are then constructed using combinations of predictors that have a large covariance with the response values (in this case the daily methane emissions measured from sheep in methane chambers), leading to a model with reliable predictive power. The PLSREGRESS function of Matlab was used to perform PLS regression with the same number of components as predictors and to plot the percentage of variance explained in the response data (chamber daily methane emissions) as a function of the number of components. It was then necessary to determine the number of components that should be used in the PLS model: using all components would result in an over-fitted model that would not fit well to an independent data set. Mean-squared errors (MSEs) for predictors and response were calculated using the PLS procedure, which includes an optional parameter for cross-validation type and the number of Monte Carlo repetitions. The method used for the cross validation of model created using the FTIR data was *k*-fold cross validation (10-fold cross validation was used in this case), which involves partitioning the original data set into equal *k* sized subsamples. All but one of these subsamples are used to train the model, with the remaining subsample being used to validate the model. All of the subsamples are used in both the training and the validation; each is used once as the validation data. This method was therefore appropriate for the data as the data set was relatively small. Mean square errors of response and prediction were plotted in order to determine the number of PLS components that should be used in the model.

## 8.3 Results

### 8.3.1 Principal components analysis

Figure 45 shows the PCA results for the FTIR spectra of the five feeds offered to animals while in methane chambers. The first two principal components accounted for 84% of the total variation within the data set (PC1=61.9%; PC2=22.1%). There was generally good grouping of feeds based on these two principal components, though there was a slight overlap between one of the perennial ryegrass samples and the grass nuts samples; these sets of samples grouped relatively closely together. It was, therefore, possible to distinguish between different feeds based on the FTIR spectra. The grass silage sample also grouped more closely to the perennial ryegrass samples than to other feeds.

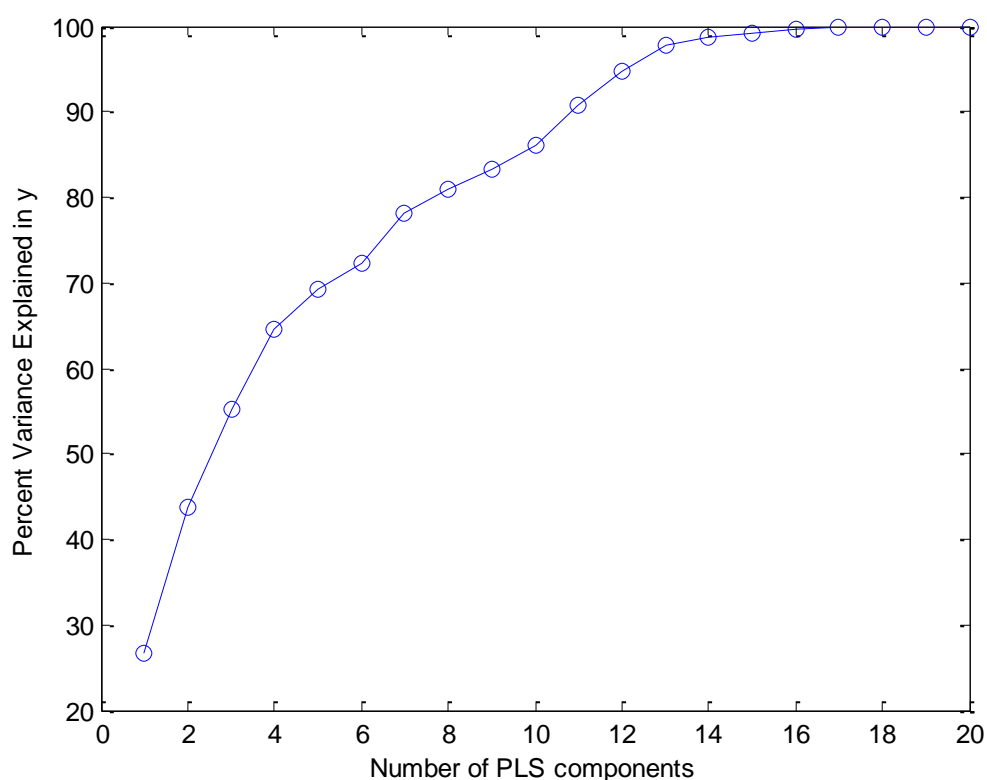


**Figure 45: Principal components analysis of feed samples using FTIR spectra**  
(1=standard silage sample, 2=perennial ryegrass, 3=permanent pasture, 4=grass nuts, 5=*Molinia caerulea*).

## 8.3.2 Partial least squares regression

### 8.3.2.1 PLS model

The results of the PLS regression showed that 100% of the variation in daily methane emissions measured in methane chambers (y) was explained by 17 components produced using the FTIR data set (x) (Figure 46). However, using all 17 components in a model would have meant that it would likely have been over-fitted and unlikely to work well with an independent data set.

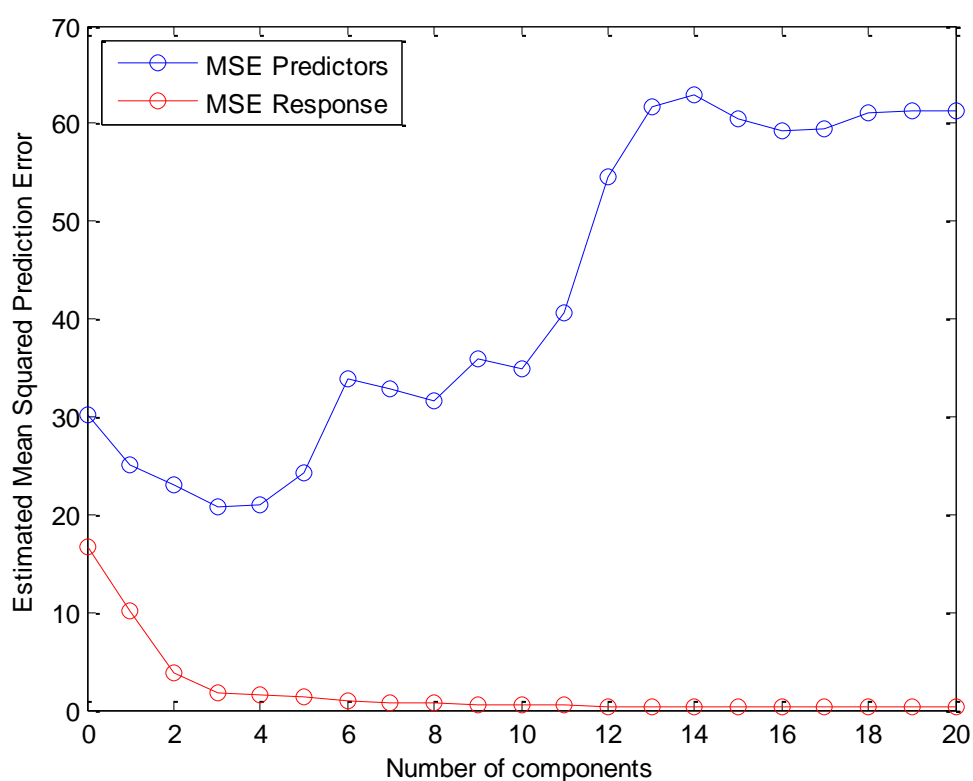


**Figure 46: Percentage of variation in daily methane emissions (measured in methane chambers) explained using PLS components of the FTIR data.**

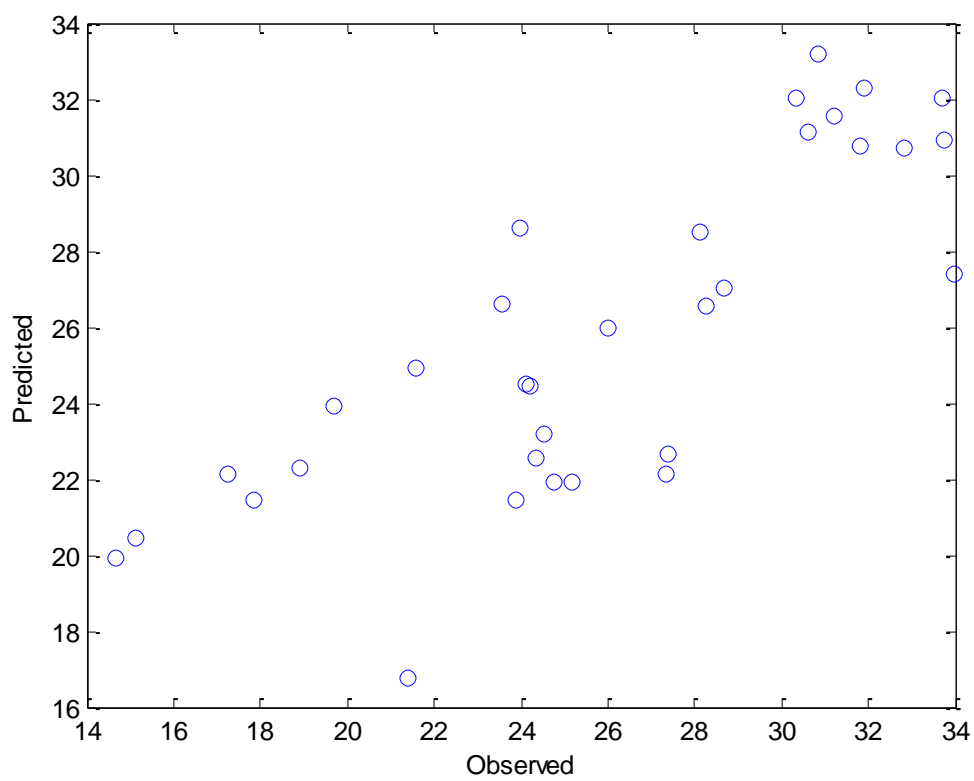
### 8.3.2.2 Cross validation of PLS model

The PLS regression using 10-fold cross validation generated MSEs of response, which show the goodness of fit of the model to the test data, and the MSEs of predictors, which show the goodness of fit of the model to the cross validation data. The MSEs of

response and predictors are plotted in Figure 47. The MSE of response is significantly reduced by 3–4 components, beyond which little improvement is evident. The MSE of prediction increases considerably after the first 3–4 components, showing divergence from the prediction model. The optimal model would therefore use approximately 4 components. Using a PLS model with 4 components, the relationship between observed values (daily methane emissions from methane chamber data) and predicted values (using FTIR data) is shown in Figure 48 ( $R=0.80$ ).



**Figure 47: Effect of increasing the number of PLS components included in the model on the estimated MSEs of predictor and response**



**Figure 48: Relationship between observed and predicted values using a PLS model with 4 components**



## **8.4 Discussion**

### **8.4.1 Principal components analysis**

The results of the PCA show that the feed samples analysed were clearly discernible based on their FTIR spectra. This suggests that FTIR spectroscopy can be used to distinguish between plant samples, which may have implications for the potential use of FTIR spectra to predict methane potentials of ruminant feeds. The results are in accordance with those of other studies, which have demonstrated that it is possible to distinguish between faecal samples of sheep fed on different diets (Moorby *et al.*, 2010; Parveen *et al.*, 2008). The perennial ryegrass and grass nuts samples, and the standard silage sample, grouped closely together. The grass nuts may have been made from a perennial ryegrass sward and the silage was made from an improved perennial ryegrass. This may explain why these samples grouped together, suggesting that the variation present in the samples was inherent to the grass species, given that the silage sample still grouped with the perennial ryegrass samples, despite having been ensiled. The PCA results demonstrate that FTIR can be used to distinguish between samples. However, to determine whether the FTIR dataset could be used to predict methane emissions from sheep, it was necessary to use the PLS regression technique.

### **8.4.2 Partial least squares regression**

The results of the PLS regression and 10-fold cross validation demonstrate that it was possible to predict daily methane emissions as measured from sheep in methane chambers using the FTIR spectra of feeds offered to these sheep. As FTIR and NIR spectroscopy are similar techniques, this is in accordance with the results presented in the Moss and Givens (2000) publication, which suggested that NIR spectroscopy was a

good predictor of ruminant methane emissions. In this experiment, as in the Moss and Givens (2000) publication, the dataset was relatively small, and larger datasets, with a wider variety of feed samples, may provide a more comprehensive idea of the factors of FTIR or NIR spectra that allow for the prediction of daily methane emissions. However, even with a relatively small dataset, the results presented in this chapter demonstrate that FTIR is reasonably good predictor of methane emissions by measured *in vivo* using methane chambers. Mid-infrared spectroscopy and PLS regression has also been used to successfully predict methane emissions from dairy cows using milk samples (Dehareng *et al.*, 2012), providing further evidence of the potential of spectroscopy techniques as proxy indicators for ruminant methane output.

While this experiment demonstrated that FTIR can be used to predict methane emissions from sheep based on their diets, the elements of the FTIR spectra that differentiated between feeds and made this prediction possible are not clear. It is likely that a combination of factors contributed to the ability of the PLS components that were predictive of methane emissions. These could include aspects of the compositional properties of the feed samples, which are likely to affect digestibility, and have been shown to be well-predicted by NIR spectroscopy (Landau *et al.*, 2006) and, to some extent, by FTIR spectroscopy (Belanche *et al.*, 2013; Belanche *et al.*, 2014). Another factor that may have contributed to the ability of the PLS components, produced using the FTIR dataset, to predict methane emissions by sheep was the identification of properties of the feed sample that may influence feed intake by sheep. Silage intake by cattle can be successfully predicted using NIR spectroscopy

(Steen *et al.*, 1998), suggesting that a factor or combination of compositional factors of the silage, which is detected by NIR spectroscopy, affects animal intake. Feed intake is known to be predictive of methane emissions (Lassey *et al.*, 1997; Molano and Clark, 2008). Therefore, if spectroscopy techniques can be used to predict feed intake, this may partially explain how spectroscopy techniques are able to predict *in vivo* methane emissions.

Previous studies have found that FTIR spectra of faecal samples from sheep can be used to differentiate between sheep based on their diets (Moorby *et al.*, 2010; Parveen *et al.*, 2008), and that FTIR of feed samples can provide information about the chemical composition of feeds (Belanche *et al.*, 2013) and some information about feed digestibilities (Belanche *et al.*, 2014). However, to my knowledge, there are no published data to date, which demonstrate the relationship between FTIR spectroscopy of feed samples and *in vivo* methane output by sheep offered these feeds.

## **8.5 Conclusion**

The rapidity with which FTIR analysis can be conducted makes it an ideal proxy indicator for methane output by sheep that could be used on a very large scale. Further work with larger data sets may provide additional insight into the factors of feeds that affect ruminant methane emissions. However, the data presented in this chapter show that FTIR spectroscopy has definite potential as a proxy indicator for methane output by sheep based on animal feed samples.

## Chapter 9

### General discussion and conclusions

## 9 General discussion and conclusions

### 9.1 Introduction

Methane is a potent greenhouse gas with a global warming potential of approximately 34 times that of carbon dioxide over a 100 year period (Stocker *et al.*, 2013).

Agriculture is a significant contributor to GHG (including methane) emissions, with small ruminant supply chains responsible for approximately 6.5% of agricultural GHG emissions (Gerber *et al.*, 2013a). Approximately 55% of the emissions from small ruminant supply chains are due to enteric fermentation (Gerber *et al.*, 2013a). There has been considerable research regarding the reduction of ruminant methane emissions, with potential methods to reduce enteric emissions including feed processing and precision feeding (Gerber *et al.*, 2013b) and increasing forage digestibility (Hristov *et al.*, 2013). However, implementing policies to reduce livestock methane emissions presents a challenge (Hegarty *et al.*, 2010), particularly given the simple and non-specific Tier 1 emissions values assumed to represent annual methane production per sheep (Bernstein *et al.*, 2007). In order to make full use of any methods for reducing methane emissions from ruminant livestock, a greater understanding of the differences between enteric methane emissions from individual animals and the factors driving these differences would be extremely useful. However, the current "gold standard" method measuring methane emissions from individual animals is the use of respiratory chambers (Chapter 2). This method is time-consuming, labour intensive, and requires the construction of chambers. Although this is possible in research facilities, this type of measurement is not practical for use in on-farm situations or on a large scale.

The overall aim of the project was to investigate, develop and validate proxies that could be used on a large scale to estimate methane output by sheep, without the need for time-consuming, labour intensive and expensive methods, such as the use of respiratory chambers. This was achieved by identifying potential proxies, which was performed by completing a review of the relevant literature (Chapter 1). Having identified the potential proxies, based on the requirement for rapidity and simplicity of data collection, the next stage was to develop methods for the use and validation of these proxies.

The three potential proxies under investigation in this project were a laser methane detector (LMD), an *in vitro* gas production technique and Fourier-transform infrared spectroscopy (FTIR). The methods and results of using these three proxies are described in Chapters 3–8. Validation of results was based on methane chamber data, which is considered to be the "gold standard" for measuring methane emissions by sheep, and is described in detail in Chapter 2. The aim of this chapter is to summarise the findings of the experiments presented in the earlier chapters of this thesis and to discuss the potential of the three proxies investigated to provide a simple and fast method for determining methane output by sheep, based on the results of the experiments performed in this project and relevant literature.

## 9.2 Laser methane detector (LMD)

### 9.2.1 Discussion

The use of the LMD as a potential means of estimating methane output by ruminant livestock is a relatively new concept (Chagunda *et al.*, 2009). The focus of the experiments carried out in this project, and of other experiments (Chagunda *et al.* 2011; 2013), has been to determine the potential of the LMD to accurately estimate methane output by animals and to begin the development of a method to use the LMD in the simplest and most time-effective way possible to achieve the desired results. The results presented in Chapters 3–5 of this thesis suggest that the LMD could potentially be used to estimate daily methane output (g) by sheep. Further work is required in order to adapt the use of the LMD to be used at a large on-farm scale. However, the results presented in this thesis suggest that further investigation of the technique is warranted. The limitations and challenges in using the LMD are described in Section 9.2.2.

Chapters 3–5 of this thesis demonstrate a novel approach to calculating daily methane emissions from animals that have undergone short periods of measurement using the LMD. This approach was used to successfully estimate daily methane emissions from animals, which were not only realistic, but also representative of data obtained from the same animals using the "gold standard" methane chamber measurements. Using this calculation method, significant positive correlations were achieved between daily methane outputs obtained using LMD and chamber data. The correlations achieved were considerably stronger than the correlation between LMD and chamber data from sheep published by Chagunda *et al.* (2013).

Despite some of the potential for inaccuracy as described in the following subsections, the LMD consistently provided a means of calculating daily methane output by sheep that not only fell within the range that would be expected but that also significantly correlated with daily methane emissions as measured using methane chambers. Given the simple method of measurement, this is an impressive feat that would justify further exploration of the technique. In addition, the LMD demonstrated potential as a tool for ranking of sheep in terms of methane production. This could potentially allow for the selection of sheep for breeding based on their rank as high or low methane producing animals (Hegarty *et al.*, 2007).

## **9.2.2 Limitations, challenges and opportunities**

### **9.2.2.1 Simplicity of method**

While the methods used to take LMD measurements during this project were uncomplicated, the problem remains that taking measurements with the LMD several times per day from individual sheep is simply not feasible at a large on-farm scale, particularly if animals are in a grazing situation rather than housed indoors.

The results presented in Chapters 5, which show LMD measurements used to calculate daily methane emissions from the group and the mean daily methane produced by each animal in the group, suggest that the method could be used on a larger scale than has previously been investigated. The data presented for group daily methane output in this thesis are not conclusive evidence that the LMD can be used in this way. However, the fact that the daily methane emissions obtained per sheep in the group measurements was on the expected scale for the animals in question, as based on



methane chamber data, suggests that the use of the LMD on a larger scale, and possibly over a greater distance, is a line of enquiry worth pursuing.

The greatest effect on LMD measurements appeared to be the time of day at which they were taken, with no significant effect between days or length of measurement. Chagunda *et al.* (2013) found that cows emitted more methane when drinking or feeding than when idle. The difference in methane emissions dependent on activity may partially explain the differences in methane emissions at different times of day; activity levels will vary over the course of each day, causing natural variation in methane output. Further research could focus on reducing the length and number of required measurements, determining to a greater extent, the measurements required to provide estimations of methane output that are representative of an entire day.

The experiments carried out using the LMD in this thesis do not use methods that are suitable for use at a large on-farm scale. However, they demonstrate the potential for use of the LMD for estimating methane output by sheep and provide evidence-based suggestions as to the direction of any further work to simplify the method of use for the LMD.

#### **9.2.2.2 Are measurements reliable and representative?**

The LMD has been shown to be accurate in its measurement of methane concentrations, both industrially, and as measured from animals in methane chambers (Chagunda *et al.*, 2009). Chagunda *et al.* (2013) found that, when used to take measurements from sheep, the sensitivity of the LMD was 93.8% and the specificity was 78.7%. However, this does not necessarily imply that LMD measurements taken using various methods can provide reliable estimates of methane emissions: Chagunda

*et al.* (2013) reported a low correlation coefficient ( $R=0.18$ ) for the relationship between LMD and chamber methane measurements taken from sheep. Even if LMD and chamber measurements correlate within certain time periods, LMD measurements taken over a short period of time may not be representative of daily (24 hour) methane outputs. Despite this, Ricci *et al.* (2013) found that models created using LMD data could successfully predict methane emissions by both steers and ewes as measured in methane chambers, and that predictions were improved by the addition of dry matter intake and body weight of animals to the model. This may be expected given that dry matter intake is a predictor of methane emissions from animals in itself (Lassey *et al.*, 1997; Molano and Clark, 2008).

The individual animal LMD measurements across all experiments described in Chapters 3–5 had a tendency to underestimate daily methane output by sheep as compared with the values obtained for the same sheep from methane chamber data. This may be due to the measurement method; methane chambers provide an enclosed space from which little expired air can escape other than through the outlet. This is not the case when taking LMD measurements; although they were taken inside a barn, the more open environment provides greater opportunity for methane produced by animals to disperse, meaning that the total methane produced may not be present in the column of gas between the animal and the LMD. Also, a small percentage of the methane produced by animals does not leave the body via the nostrils or mouth but via the anus. Directing the LMD towards the nostrils of the sheep would not allow measurement of any methane leaving the sheep via the anus. In chambers, there would be no discrimination between methane output from different

orifices; all, or the vast majority of gases released by the sheep would leave the chamber via the outlet.

The tendency of the daily methane emissions estimated using the LMD to underestimate those produced by the methane chamber data is probably due to a combination of the factors detailed above and, potentially, the calculation method for LMD daily methane output (Section 9.2.2.3).

### **9.2.2.3 Daily methane calculations**

The daily methane calculations used to calculate daily methane emissions using the LMD data from both individual sheep and groups of sheep were based on a number of assumptions. The body weight of each sheep was assumed to have a linear relationship with the tidal volume, and the number of breaths per minute (scaled up to breaths per day) was kept as a constant value. The respiratory rate (L/d) of each sheep was, therefore, an estimate and subject to error. However, from the perspective of keeping the method as simple as possible, it was not practical to attempt to measure daily respiratory rates for each individual animal.

As discussed in Chapter 3, an attempt was made to separate eructation peaks and normal breathing methane concentrations in the LMD data and to use different volumes (i.e. eructation volumes and respiratory rates) to provide more accurate daily methane calculations. The main problem presented was establishing an eructation volume to use as a constant for this purpose. Eructation volume has potential to vary enormously both between and within individual sheep and there is an apparent lack of literature that defines average eructation volumes for sheep of varying sizes. Without data of this type, the method of separating the eructation peaks and normal breathing

methane concentrations merely adds to any inaccuracies that are already present in the calculation method.

Throughout all experiments on the use of the LMD presented in this thesis, the daily methane emission calculated using LMD measurements tended to underestimate daily methane emissions as calculated from methane chamber data. To compensate for any underestimation of methane emissions calculated using the LMD data compared with chamber data, correction factors were calculated for each sheep to scale the LMD data to the chamber data. In the two larger scale experiments detailed in Chapters 4 and 5, the mean correction factor required was similar between experiments (1.18 and 1.24). However, in each experiment, there was a wide range of values for required correction factors, making it difficult to determine whether alterations to methods of calculation, such as increasing the assumed breathing rate and tidal volume, could improve the relationship between LMD and chamber measurements of daily methane emissions.

The method presented in this thesis for calculating daily methane output from individual and group LMD measurements is a novel approach to using LMD measurements to estimate methane output from sheep. With additional information about the volume of individual eructations, the accuracy of calculations could be improved. However, the method of calculation provides a simple means of estimating methane emissions from individual sheep and could be the basis of calculations to calculate group, or even herd methane emissions.

### **9.2.3 Conclusions and recommendations**

It is possible to make a number of conclusions and recommendations based on the LMD measurements detailed in Chapters 3–5. Some have been discussed in

Section 9.2.2; the aim of this section is to summarise the conclusions and recommendations for further work.

Although the use of the LMD for measuring methane output by ruminants is very much in a developmental stage, the results presented in this thesis demonstrate real potential for the LMD as a future proxy for methane output by sheep. The challenges faced in developing the LMD as a proxy that could be used at a large on-farm scale are: whether the LMD can provide realistic and representative estimates of methane emissions by sheep; and the simplification of the measurement method to produce a protocol that could be used on farm and in different situations (i.e. grazing, housed animals). Developing a method to calculate daily methane emissions based on LMD data is another challenge, which has been attempted in this thesis in a novel manner. There are potential improvements to the method presented, which could be implemented with sufficient supporting information. Recommendations for further investigation into the use of the LMD are as follows:

1. To pursue the idea of taking LMD measurements from groups of sheep (as in Chapter 5), incorporating different situations (i.e. whether animals are grazing or housed) and testing the distance from which the LMD can provide reasonable estimates of methane output.
2. To further investigate the length and frequency of LMD measurement periods, determining the minimum times and frequencies possible, creating the simplest and least labour intensive method possible.

3. To improve and develop the presented method for calculating daily methane output using LMD measurements, based less heavily on assumptions and more on measured values.

## 9.3 *In vitro* gas production

### 9.3.1 Discussion

*In vitro* gas production is a well-established method of determining the digestibility of ruminant feed samples using a quick and simple laboratory technique. There are numerous variations on the method used; in this case, the method used throughout the experiments detailed in Chapter 6 and 7 was based on that of Theodorou *et al.* (1994). The aim of the gas production experiments was to compare methane production potentials of different feeds and upland plants and to determine whether their *in vitro* methane production profiles could predict their methane production when fed to sheep.

The results presented in Chapter 6 and 7 demonstrate that, not only can the *in vitro* gas production technique be used to differentiate between plants in terms of their methane potentials, but also that there were significant positive correlations between methane production per gram of apparently digested DM and per gram of DM using the *in vitro* method and those obtained using the *in vivo* methane chamber experiments. The gas production technique, therefore, has the potential to be used as a proxy to predict methane emissions from different animals given different diets, provided a reasonable estimate of the DM intake of animals can be made. It should also be noted that, when two upland plants were mixed in varying proportions, the mixtures, which varied by only 20% were easily distinguishable using the technique: this suggests a high level of accuracy within runs of the gas production technique.

## 9.3.2 Limitations, challenges and opportunities

### 9.3.2.1 Differences in rumen inoculum

As discussed in Chapter 6, the gas production technique requires inoculation of samples with rumen fluid. This presents a difficulty as, when rumen fluid is collected from fistulated animals, it must be kept at 39°C to imitate the rumen environment. Even when kept at this temperature, rumen fluid cannot be kept for use on multiple runs of the protocol. However, rumen fluid taken at different times will naturally vary, which may have an effect on the *in vitro* gas production parameters. To reduce the impact of differences in rumen inoculum, the fluid should be collected at the same time of day from the same animals, and a mixture of rumen fluid from several animals should be used (Mauricio *et al.*, 1999). However, as shown by the comparison of standard samples in Chapter 7 of this thesis, there can still be significant variation in methane produced between runs of the experiment. It is, therefore, important that a standard sample is used between runs in order to allow correction for differences in rumen fluid.

In itself, obtaining rumen fluid may present a problem as it requires either fistulated animals or stomach tubing, both of which are subject to Home Office regulation and require careful management. However, if the technique were to be used as a means of predicting methane potentials of diets from farms, the samples would be collected on-farm and sent to a research facility for analysis; this would also reduce any variation in results due to laboratory conditions and staff performing analyses.



#### **9.3.2.2 DM intake**

The *in vitro* gas production technique is used to measure the methane produced by one gram of dry matter in the system. This may be multiplied by dry matter intake in order to obtain a prediction of daily methane production by animals. However, estimating DM intake may not be particularly simple: for example, in grazing situations, particularly if animals are grazing in upland environments rather than pastures, it is difficult to monitor the intake of animals. The DM of grazing material will also be subject to change depending on weather and even time of day. In situations where animals are housed in groups, DM intake for each individual animal is also difficult to measure, though a reasonable estimate of group DM intake can be obtained provided that the weight and DM of offered feed and an estimate of refused feed is monitored.

The challenge posed by measuring DM intake may limit the usefulness of the *in vitro* gas production technique, particularly as DM intake does not correlate particularly well with body weight (Lassey *et al.*, 1997). However, in cases where animal intake is already monitored, taking samples to be used for *in vitro* gas production analysis would be easily manageable.

#### **9.3.3 Conclusions and recommendations**

The results presented in Chapter 7 suggest that *in vitro* gas production can, to an extent, predict methane emissions by sheep based on the *in vitro* methane production profile of their feeds. There is variation between animals in terms of their methane production, which cannot be attributed to differences in diet and DM intake. However, it is well established that there is a strong relationship between DM intake and

methane output. Diet is also known to affect methane production in the rumen; a method of estimating methane emissions that uses both DM intake and methane potentials of feeds is likely to provide accurate measures of methane output.

Use of the gas production technique to estimate methane output by sheep is not without limitations, as discussed in Section 9.3.2. However, as a means of predicting the effect that different diets will have on methane output, the gas production technique has been shown in this thesis to be effective.

Further work should focus on creating a larger dataset of feeds given to animals in methane chambers that have also been analysed using the *in vitro* gas production technique. This could confirm the ability of the technique to predict methane output from sheep based on their diets and DM intakes.

## 9.4 Fourier-transform infrared (FTIR) spectroscopy

### 9.4.1 Discussion

Fourier-transform infrared (FTIR) spectroscopy is a rapid-throughput laboratory technique that requires a very small amount of plant or feed material (Allison *et al.*, 2009), making it ideal as a proxy indicator for methane output by sheep provided that it can be used to successfully predict methane emissions using feed samples or faecal matter. The data presented in Chapter 8 of this thesis demonstrate that it is possible to distinguish between different feed samples on the basis of their FTIR spectra. Furthermore, there is potential for FTIR spectra of feed samples to successfully predict daily methane emissions from sheep as measured in methane chambers. Although a study has demonstrated that NIR spectroscopy of feed samples can predict ruminant methane emissions (Moss and Givens, 2000), and FTIR of milk samples from dairy cows can be used to predict methane emissions (Dehareng, 2012), this is, to my knowledge, the first example of FTIR spectroscopy of feed samples being used to predict methane emissions from sheep.

The main limitation of the data presented in this thesis is that the data set is relatively small compared with data sets presented in the literature: for example, Belanche *et al.* (2013) used 663 samples, representing 80 feed types. Conclusions are therefore limited from this small data set regarding the aspects of the FTIR spectra that enable the prediction of methane emissions. Further work should, therefore, involve using larger data sets, with contrasting feed samples, to perform a similar analysis. This may provide a more comprehensive idea of the properties of feeds that cause animals to

produce more or less methane, whether this is related to feed composition, or perhaps whether certain properties of feeds affect feed intake by animals.

## 9.5 General discussion

While further work may be required to optimise the methods used to estimate or predict methane output by sheep, the data presented in this thesis provide evidence for the potential of three proxy indicators for this purpose. All data collected were validated against data produced using respiratory chambers, which is considered to be the "gold standard" measurement method for methane output by sheep.

The use of the LMD is a novel method for the estimation of methane emissions by ruminants. Chagunda *et al.* (2009) published the first evidence that the LMD could be used for this purpose rather than its original purpose of detecting industrial gas leaks. Since this publication, other studies have added to the evidence that the LMD can successfully be used to estimate methane output by ruminants, although this work has focused largely on cattle (Chagunda *et al.*, 2011; Chagunda and Yan, 2011; Ricci *et al.*, 2012). One publication also discusses the use of the LMD for estimating methane emissions from sheep (Chagunda *et al.*, 2013). However, the methods for the use of the LMD and the calculation of daily methane emissions presented in this thesis are novel.

In vitro gas production and FTIR spectroscopy are rapid laboratory methods which require only small amounts of sample (Theodorou *et al.*, 1994; Allison *et al.*, 2009) and, as such would be ideal for large scale estimation and prediction of methane emissions from sheep using samples of feed offered to animals. The data presented in Chapters 6–8 demonstrate that there is real potential for these methods to be used for this purpose, although further validation of the techniques is necessary.

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## **Supplementary appendix: LMD measurements of methane concentrations from wallabies and goats at Borth animalarium**

### **Introduction**

The digestive system of macropod marsupials (kangaroos and wallabies) is similar to that of ruminants in that they are both foregut fermenters. However, macropod marsupials appear to produce negligible quantities of methane in comparison to ruminant livestock. Kempton *et al.* (1976) found that grey kangaroos did not expire methane in breath and released very little from the anus. Von Engelhardt *et al.* (1978) compared methane production in sheep and Tammar wallabies fed on the same diet, finding that Tammar wallabies produced 6.5 to 11ml of methane per kilogram of body weight per hour. This amounted to 1-2% of digestible energy, considerably less than the methane produced by sheep.

More recently, Wilson and Edwards (2008) suggested that, by using kangaroo meat as an alternative to meat from ruminant livestock, Australia's methane emissions could be reduced. There is little evidence of prolific numbers of methanogenic archaea in the foregut of macropod marsupials (Evans *et al.*, 2008; Maguire and Ouwerkerk, 2008).

This may indicate that the only methane produced by these animals occurs in the hindgut, which would explain why Kempton *et al.* (1976) found no methane in breath, only from the anus. Madsen and Bertelsen (2012) conducted a study in which red-necked wallabies (*Macropus rufogriseus*) were placed in open-circuit respiration chambers, which are commonly used to measure methane production by ruminants. In this way, any methane from either the foregut or hindgut of the animals would be

measured. The study concluded that red-necked wallabies did produce methane, but only 25-33% of the methane that would be expected from ruminants fed on the same diet.

The laser methane detector (LMD; Tokyo Gas Engineering Ltd., 2006) has been used to detect methane from ruminant livestock (Chagunda *et al.*, 2009, 2011, 2013; Ricci *et al.*, 2012, 2013). These studies have found that the LMD is sensitive enough to detect both eructation peaks and normal breathing concentrations, when the laser is directed at the nostrils of ruminant livestock. Chagunda *et al.* (2013) found that the sensitivity of the LMD was greater than 79%. It can, therefore, be assumed that the LMD can provide a reliable indication of whether or not an animal produces methane.

## **Aims and objectives**

The aim of this study was to determine whether the LMD could detect any methane produced by Parma wallabies. The objectives were as follows:

1. To take LMD measurements from individual wallabies at Borth animalarium.
2. To take LMD measurements from individual goats at Borth animalarium in order to represent ruminant methane emissions and provide a comparison for the LMD data taken from wallabies.

## **Materials and methods**

### **Animals**

The animals used were Parma wallabies (*Macropus parma*) and pygmy goats at Borth Animalarium. Borth Animalarium has a mob of between five and ten Parma wallabies. The pygmy goats, also at Borth Animalarium, were used as a comparison as they are ruminants and are known to produce methane.

### **Diet**

As the animals were housed in groups and are frequently fed by visitors to the animalarium, it was not possible to accurately measure their dry matter intake. However, both wallabies and goats were offered diets consisting of fresh fruit, nuts and vegetables (including lettuce, broccoli, cabbage, carrot, grape, peanuts). Wallabies were also offered pony nuts and goats were offered a sheep and goat mix (further details were not available upon request). Animals had free access to water.

### **LMD measurements from wallabies**

LMD measurements were taken from two individual wallabies by pointing the LMD at the nostrils from a distance of approximately one metre away. Measurements were taken for between one and two minutes: the time of measurements was limited as wallabies were able to move within their enclosure freely. Measurements were taken using the procedure described in Section 3.2 of this thesis and corrected for background methane as described in Section 3.2.5. Background measurements were taken prior to wallaby measurements by pointing the LMD at fences and empty

enclosures. There appeared to be relative high levels of background methane at Borth animalarium, suggesting that it was necessary to correct for background methane.

### **LMD measurements from goats**

Individual LMD measurements were also taken from the nostrils of two goats from approximately one metre distance, using the procedure described in Section 3.2.

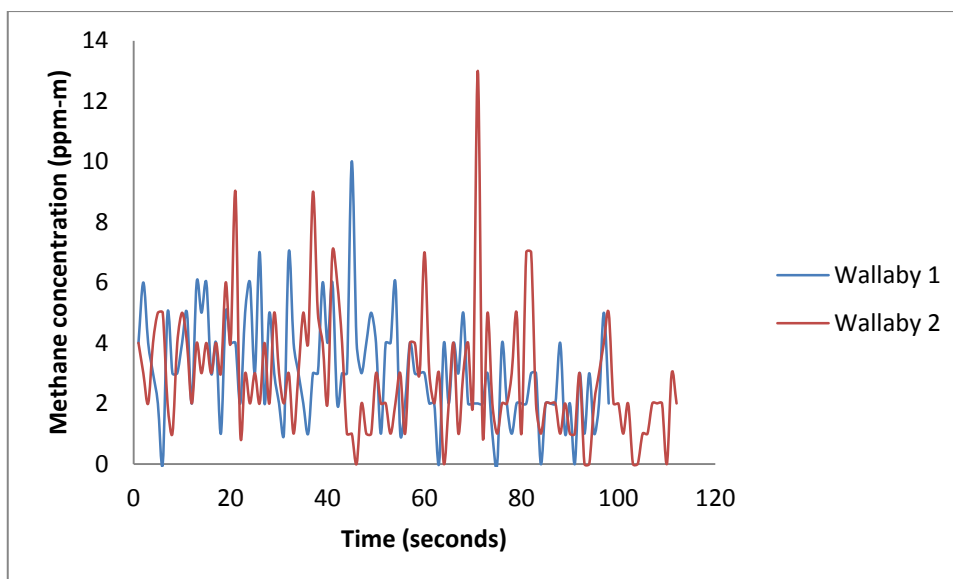
Measurements were corrected for background methane as described in Section 3.2.5.

Times of measurement periods were limited by animal movement as they were able to move within their enclosure freely. However, measurement periods were at least 100 seconds.

## **Results**

### **Wallaby measurements**

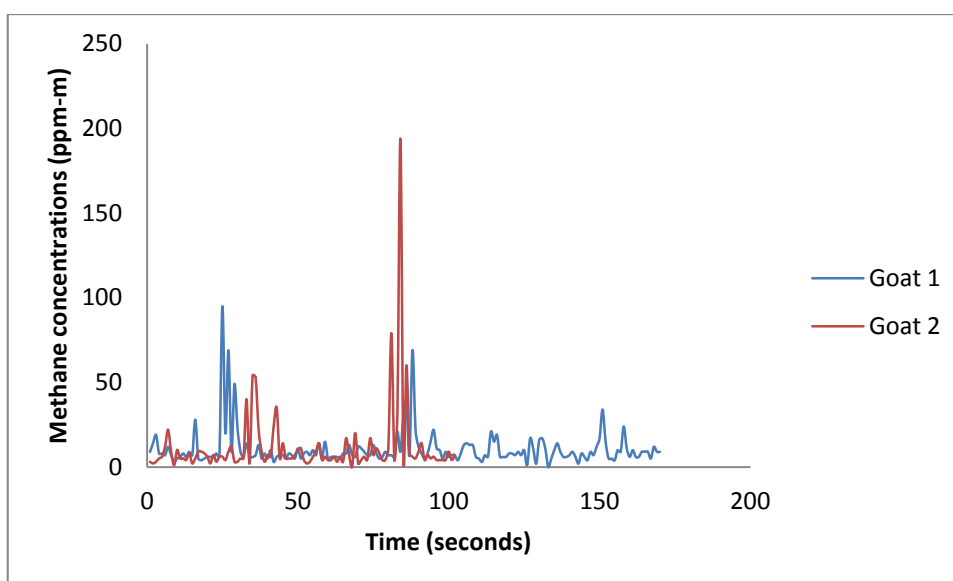
The LMD outputs for individual measurements (corrected for background methane) from wallabies are shown in Figure 49. Although there is fluctuation over time, methane concentrations measured did not exceed 14ppm-m, with mean values of 3.1 and 2.9ppm-m from wallaby 1 and wallaby 2, respectively.



**Figure 49: LMD output (ppm-m) from wallaby measurements**

## Goat measurements

The LMD outputs for individual measurements (corrected for background methane) from goats are shown in Figure 50. Clear eructation peaks are shown. Mean methane concentrations during measurement periods were 10.7 and 11.6ppm-m for goat 1 and goat 2, respectively.



**Figure 50: LMD output (ppm-m) from goat measurements**

## Comparison of wallaby and goat LMD measurements

Wallaby and goat measurements are plotted on the same axes in Figure 51, showing the clear differences between species in terms of LMD outputs. Not only were mean methane concentrations considerably higher from goats than from wallabies, but eructation peaks are clearly shown in the measurements taken from goats. There is no evidence of eructation peaks in the measurements taken from wallabies.

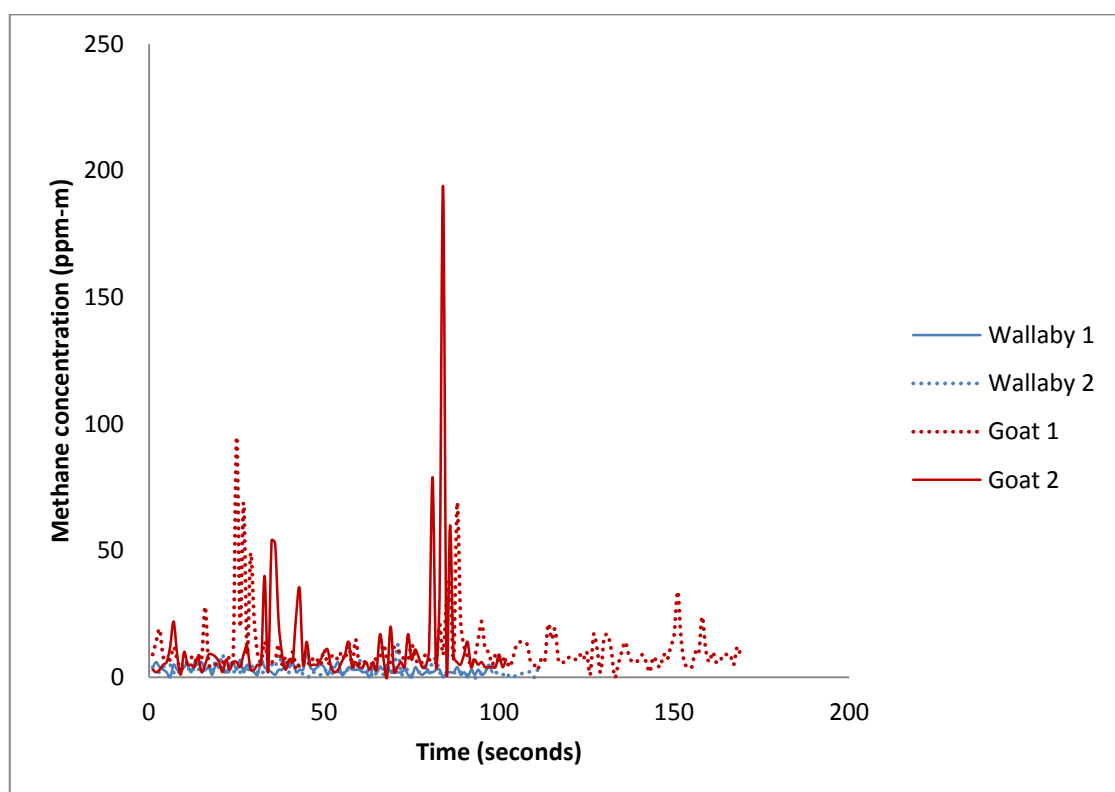


Figure 51: LMD measurements from wallabies and goats (ppm-m)

## Discussion

The data presented here were limited in quality due to the nature of the measurements taken. Measurements were taken at Borth animalarium and were able to move free within enclosures and, although all animals were accustomed to human



contact, this presented a difficulty in obtaining measurements and limited the time of measurements. Despite the limitations, clear differences were shown between methane concentration measurements taken from wallabies and those taken from goats. The mean methane concentration measured from wallabies was approximately 3ppm-m compared with a mean of approximately 11ppm-m measured from goats. Clear eructation peaks were also shown in LMD measurements from goats, whereas there was no evidence of eructation peaks in the LMD measurements taken from wallabies.

The LMD data suggest that some methane is produced by wallabies, which is consistent with previous studies (Kempton *et al.*, 1976; Von Engelhardt *et al.*, 1978; Madsen and Bertelsen, 2012) that have found that kangaroos and wallabies produce methane, albeit on a small scale compared with ruminant methane emissions.

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